Expression of the Sensory Neuropeptide Encoding Genes, βPPT-A and α-CGRP in Adjuvant-induced Joint Inflammation

Duncan George Spencer Bulling B.Sc.

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DECLARATION

I declare that this thesis was written entirely by myself and represents work that was conducted by myself except for the following procedures detailed below.

1) Recordings from small diameter sensory nerves innervating the rat knee joint injected with FCA were conducted by David Kelly.

2) Sectioning of tibio-tarsal joints and subsequent staining was performed by the research group of Dr. Donald Salter, Department of Pathology, University of Edinburgh.

3) Radioimmunoassays for SP and CGRP conducted on cultures of DRG neurones treated with antisense oligonucleotides were perfomed by John Bennie and Shona Carroll of the Medical Research Council Brain Metabolism Unit, Royal Edinburgh Hospital, Edinburgh.

4) Cycle sequencing of PCR products was performed by Val Lyons.

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ABSTRACT

Small diameter sensory nerves are implicated in the aetiology of inflammation; their cell bodies are called dorsal root ganglia (DRG). Within 30min of induction of unilateral tibio-tarsal joint inflammation by injection of Freund's Complete Adjuvant (FCA) there is an increase in the mRNAs of β preprotachykinin-A (β PPT-A), which encodes the sensory neuropeptides substance P and neurokinin A, and the α-calcitonin gene-related peptide (CGRP) encoding gene, in DRG of innervating small diameter nerves. The increases in both transcripts per small diameter neurones peaks at 1h, and is sustained at levels significantly above untreated controls up to 8 h after injection. An increase in βPPT-A hetero-nuclear RNA, measured using a probe complementary to a sequence to intron E, is seen within 30 min of FCA injection, indicating the initial increase in mRNAs encoding this gene is at least in part due to increased transcription. Increased expression per small neurone is augmented by an increase in the proportion of small diameter cells expressing the mRNAs of both βPPT-A and α-CGRP ipsilateral to FCA injection at 1h, and maintained at subsequent time points. Over the same time course the proportion of large diameters neurones expressing βPPT-A and α-CGRP encoding mRNA did not change, however increases of both transcripts per neurone increased within 30 min, peaking at 1h and returning to control levels at subsequent time points. It is possible that the initial increases in transcripts per small and large diameter neurones are attributable to a common molecular mechanism.

Previous experiments have implicated the transcription factor AP-2 in playing a role in the inflammation-induced upregulation βPPT-A and α-CGRP. However the speed of increases in transcripts coupled with the failure of the pre-administration of cycloheximide to prevent the increases in α-CGRP encoding mRNA 30 min after adjuvant injection indicates that increased transcription is independent of *de novo* protein synthesis. Hence it is likely that the transcription/translation of immediate early genes, such as AP-2 is not involved in the initial upregulation of βPPT-A and α-CGRP in response to FCA injection.

Increases in mRNAs are initially mirrored in increases in the encoded peptides, substance P and CGRP. Significant increases in both peptides occur within 30 min of injection, peaking at 1h. At subsequent time points peptides fall to control levels with the exception of SP levels at 8h, which are significantly below control levels, this may indicate increased axonal transport of peptide away from the DRG. The fast increase in both gene expression and peptide levels, implicate SP, NKA and CGRP in playing roles in the early aetiology of FCA induced inflammation.

Within 15 min of injection of FCA around the knee joint there is an increase in action potentials in innervating small diameter nerves within the range of the response of polymodal C fibres to capsaicin application. Furthermore prior administration of local anaesthetic around the sciatic nerve ipsilateral to adjuvant injection prevented increases in expression of βPPT-A and α-CGRP mRNAs suggesting that an increased neural activity in response to FCA injection may underlie their upregulation.

In the first 8h of FCA induced inflammation, oedema formation and mechanical hyperalgesia develop swiftly (within 30 min) with a progressive nature. However infiltration by inflammatory cells is limited to the site of injection within 8h and up to 24h after FCA administration. No changes were noted in bone, joint or synovial tissue.

Application of phosphorothioate oligonucleotides increases production of SP and CGRP in cultures of adult DRG neurones in a dose dependent manner, suggesting stress may regulate expression of their encoding genes. The NFκB and STAT families are two classes of transcription factors implicated in signalling stressful stimuli. The are also capable of modulating rapid increases in gene expression due to post-translational activation by phosphorylation. Analysis of the promoter regions of both βPPT-A and the α-CGRP encoding gene revealed a number of sequences sharing homology with NFκB binding sites, interferon-γ activated site (GAS) and interferon-stimulated response element (ISRE), the latter two are bound by members of the STAT family. Western blot analysis revealed the presence of IκB-α, a member

of the family of NFκB inhibitors, in DRG extracts. By association members of NFκB family will also be present in DRG. Sequencing of PCR products generated from cDNA derived from DRG mRNA and primers designed to amplify STATs 1, 3 and 4 demonstrated that STAT 1 is expressed in DRG. The activational state of either STAT 1 or members of the NFκB family is unclear.

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1:INTRODUCTION

1.1 Joint Inflammation: an overview

In humans, joints affected by injury or inflammatory articular diseases, such as inflammatory or degenerative disorders, show hyperalgesia, a decrease in the pain threshold and an exaggerated response to suprathreshold stimuli (Treede et al 1992), and persistent pain in the joint area (Lewis 1938, 1942, Kellgren 1939, McEwen 1943, Obletz et al 1949, Kellgren and Samuel 1950). Hyperalgesia and pain are aggravated by movement and mechanical stimulation of the affected limb (Kellgren 1939, McEwen 1943). The sensation of pain, termed nociceptive pain, is caused by high intensity stimuli with potential to cause tissue damage.

Joint pain in humans can be created experimentally by application of noxious mechanical, thermal and chemical stimuli to the fibrous structures of the joint, ligaments and joint capsule (Lewis 1942, McEwen 1943, Kellgren and Samuel 1950).

The three major networks in the body, namely the immune system, endocrine system and nervous system, are implicated in the development of joint inflammation. In order to study the role of these systems in the pathogenesis of experimental joint inflammation, there is a need for an *in vivo* system. As a result a number of animal models of experimental joint inflammation have been developed with a view to designing therapeutic strategies against inflammatory joint disorders.

Injection of urate crystals or kaolin and/or carrageenan into the cavity of a joint in dogs, cats, rabbits, rats and pigeon causes an acute inflammation (Faires and McCarty 1962, Rosenthale et al 1966, Brune et al 1974, Schumacher et al 1974, Santer et al 1983, Okunda et al 1984, Schaible and Schmidt 1985, 1988, Schaible et al 1987, Coderre and Wall 1987). Carrageenan injection causes the development of

hyperalgesia in response to mechanical stimuli within 1-3h (Santer et al 1983). In addition there is formation of swelling, or oedema, caused by synthesis and release of inflammatory mediators (Moncada et al 1979, Sedgwick and Willoughby 1985), and a rapid response in the immune system, with cellular infiltration by polymorphonuclear granulocytes at the site of inflammation (Santer et al 1983).

Rat models involving the injection of Freund's complete adjuvant (FCA), a suspension of heat killed bacteria (typically *Mycobacterium tuberculosis* or *M. butyricum*) have been used to study inflammation in joints. Injection of FCA into the footpad, tailbase or lymph nodes instigates a two stage inflammatory reaction. Initially there is a local acute inflammatory reaction within the first few hours of injection, which subsides after 3-5 days. In the second week following injection inflammation occurs in the distal joints of limbs, and also other sites in the body including eyes, ears, tail and genitalia (Pearson 1963, Billingham and Davies 1979). The inflammation subsides after 4 weeks but may recur spontaneously.

Observations that animals which have been subjected to whole body irradiation have a delayed appearance of joint swelling (Wakeman 1960) suggest that the pathogenesis of the disease has, at least in part, an immunological basis. Further investigation suggested the aetiology of the model involves the immunogenic recognition of a specific epitope of the mycobacterial 65kDa heat shock protein, hsp 65, causing activation of host T-cells (Van Eden et al 1985). Bacterial hsp 65 shows a degree of homology with mammalian heat shock protein 60, hsp 60, (Anderton et al 1995) and also a self antigen in joint cartilage (Van Eden et al 1985). As a result, T-cells initially activated against hsp 65 cross-react with host proteins, including cartilage proteoglycans (Van Eden et al 1985), inducing an auto-immune disease state (Van Eden et al 1989). Rheumatoid arthritis (RA) is also characterised by an auto-immune

reaction, and FCA induced inflammation has been proposed as an animal model for RA under the name adjuvant arthritis. There are a number of biochemical and pathological similarities between RA and adjuvant arthritis, as summarised by Rainsford (1982), although the absence of circulating rheumatoid factor in adjuvant arthritis is perceived as weakness as a model for RA.

Study of the progression of adjuvant arthritis is complicated by the occurrence of systemic effects including reduced weight gain (Pearson and Wood 1959), increased vocalisation and irritability (Colpaert et al 1982), hyperventilation (Colpaert and Van Hoogen 1983), reduced mobility (de Castro Costa 1987), as well as chronic pain (Colpaert 1987) and activation of the hypothalamic-pituitary-adrenal axis (Sarlis et al 1992). By reducing the amount of mycobacteria injected it is possible to attain a less severe arthritic condition, without the complicating effects of systemic disease (Butler et al 1992). Injection of 100-150μg of mycobacterium into the foot pad or skin overlying the tibio-tarsal joint leads to the development of a discrete monoarthritis restricted to the injected joint. The monoarthritic state has also been referred to as unilateral adjuvant-induced arthritis. Injection causes an acute inflammation at the site of injection lasting between 2 and 4 weeks, but abolishes (Iadarola et al 1988 a and b) or severely limits (Grubb et al 1991) the appearance of inflammation in distal joints.

The formalin test, first described by Dubuisson and Dennis (1977) is another model for acute nociception and inflammation. Subcutaneous injection of diluted formalin into the hindpaw of rats and mice produces a biphasic pain-related behavioural response within the first hour after injection, accompanied by a long lasting inflammation of the paw (Tjolsen et al 1992). The early phase, occurring immediately after injection and lasting 5-10 min, is thought to be due to direct chemical stimulation. The late phase, between 15 and 60 min post injection, is a result of

inflammation. (Tjolsen and Hole 1997).

Work presented in this thesis employed FCA-induced unilateral inflammation of the rat tibio-tarsal joint as described in Donaldson et al 1993.

1.2 Sensory Nervous System

The sensory nervous system has been implicated in playing a role in the aetiology of inflammatory joint disease. This is based on the observation that in conditions associated with a loss of innervation to a joint, such as poliomyelitis (Glick 1967) or hemiplegia, (Thompson and Bywaters 1962) there is a resultant protection of the joint from rheumatoid arthritis. Additionally hyperalgesia, swelling and joint destruction in experimental inflammation in the rat induced by injection of *M butyricum* can be attenuated by peripheral nerve section (Levine et al 1986), however a subsequent study failed to find an attenuation of adjuvant arthritis after surgical denervation(Ahmed et al 1995b).

1.2.1 Innervation of tibio-tarsal joint

Sensory innervation of the rat tibio-tarsal joint is provided by the tibial nerve, a branch of the sciatic nerve. Sensory nerve fibres within the sciatic nerve are pseudounipolar, having a peripheral branch innervating the joint and associated structures, and a central branch, which enters the spinal cord at the dorsal tip of the dorsal horn, branching and projecting to nuclei in the spinal grey matter and brain stem (Greene 1959, Kelly 1991). The cell bodies, or somata of sensory neurones are found in a structure known as the dorsal root ganglia (DRG). The cell bodies contain the nucleus and organelles of the neurone, and are the site of production for a large number of enzymes and bioactive peptides. The latter include neuropeptides, a class of small peptides which are thought to act as neurotransmitters. Primary sensory

nerves relay sensory information between the periphery and the central nervous system.

The cell bodies of the sciatic nerve are located in the lumbar 4,5 and 6 dorsal root ganglia, L4-L6 DRG, (Greene 1959). Only L5 DRG are solely innervated by the sciatic nerve; L6 derived nerves also contribute to the make up of the pudendal plexus, whereas L4 nerve fibres are found in obturator and femoral nerves (Greene 1959).

1.2.2 Sensory nerve population and distribution

The sciatic nerve contains a variety of groups or phenotypes of neurones, which have been classified by a number of features as discussed below. Analysis of neuronal cell bodies by diameter and staining the cytoplasm with Nissl stain has revealed 2 major classes of DRG neurones; large light (A) and small dark (B) cells (Andres 1961). However subsequent studies have shown that there is a size overlap between the light and dark subpopulations (Lawson 1979, Lawson et al 1984). A refinement of the division of primary sensory neurones has been proposed based on fibre diameter and conduction velocity, this is summarised in table 1.1 below.

Fibre Class	Conduction Velocity (m/s)	Mean Cell Size ±SD (μm^2)	Mean Cell Diameter (μm)
Aα (Ia and b)	>30	1171±410	39±23
Aβ (II)	14-30	1104±310	38±20
Aα/β	>14	1142±366	38±22
Aδ (III)	2.2-8	702±448	30±24
C (IV)	<1.4	449±148	24±14

Table 1.1 Classification of sensory nerve fibres in adult rat by their conduction velocity from the sciatic nerve to the DRG, cross sectional area and diameter of DRG neurone. The latter assuming that neurones are circular in cross section, from Harper and Lawson (1985)

Aδ and C fibres terminate as free nerve endings in various areas of the joint tissue; the fibrous capsule, adipose tissue, ligaments, menisci, and periosteum (Samuel 1952, Sklenska 1965, Frommer and Monroe 1966, Polacek 1966, Freeman and Wyke 1967, Klineberg et al 1971, Halata et al 1984). Following an initial report describing free nerve endings of Aδ and C fibres in the synovial layer of joints (Polacek 1966) subsequent studies failed to find them in this area (Samuel et al 1952, Freeman and Wyke 1967, Halata et al 1984), however immunhistochemical studies from humans have demonstrated the presence of free nerve endings in the synovium (Mapp et al 1990).

Electron microscope studies of free nerve endings in the cat knee joint capsule show that Aδ and C fibres form terminal trees (Heppleman et al 1990). These take on a 'string of beads' appearance with spindle-shaped thick segments (the beads) connected by thinner segments. It is proposed that these beads and the end bulb of the

nerve form the receptive sites of sensory endings.

Studies of response of the sensory innervation of the knee joint to innocuous and noxious mechanical stimuli and chemical stimuli have identified different classes of receptor units (Messlinger 1996). Units which respond to both mechanical and chemical stimuli can be regarded as bimodal nociceptors, similar to polymodal nociceptors found in cutaneous tissue (Lang et al 1990). Based on their response to different mechanical stimuli articular afferents have be divided into groups: low-threshold units, which respond to normal passive movements and high-threshold units, which respond to noxious movements, or show no response to movement, but do respond to pressure applied to the receptive field (Schaible and Schmidt 1983 a and b, 1985). Studies of the conduction velocities of these two different classes of units revealed that low threshold units are distributed throughout the range of conduction velocities, being especially prevalent in the faster conducting nerves (Aβ). The high threshold units belong to the classes of slow conducting nerves, implicating Aδ and C fibres in the transmission of noxious information.

Studies of the central terminals of sensory afferents in rat, cat and non-human primates have shown that the different classes of fibres innervate different areas of the spinal cord. Aδ and C fibres terminate mainly in laminae I and II, although Aδ also innervate lamina III. Lamina III also receives input from Aβ fibres, as does lamina IV (Jessell and Dodd 1989).

Sensory neurones display a wide variety of different neurochemical markers. Calbindin D28k, calcitonin gene-related peptide (CGRP), cholecystokinin (CCK) and vasoactive intestinal peptide (Tuchscherer and Seybold 1985, Ju et al 1987, Villar et al 1989, Antal et al 1990, Ishida-Yamamoto and Senba 1990, Noguchi et al 1990) are found in both large and small cells. However other neurochemical markers are found

in cell populations with more restricted size. Substance P (SP), somatostatin (SOM), galanin, and fluoride-resistant acid phosphatase (FRAP) are usually found in small B cells (Price 1985, Kai-Kai et al 1986, Ju et al 1987), whilst parvalbumin (PV), carbonic anhydrase (CA) and GM1 ganglioside are restricted to large A cells (Wong et al 1987, Robertson and Grant 1989, Antal et al 1990). Some of these markers are widely distributed, for example CGRP is found in approximately 50% of DRG neurones (Ju et al 1987), whereas other neurochemicals have far more limited distributions, for example the neuropeptide galanin being only present in approximately 5% of neurones (Villar et al 1989). Evidence has come to light of a number of complex coexistence relationships between these markers, especially in small cells. CGRP coexists with SP, SOM, CCK, galanin and FRAP (Ju et al 1987, Gibbins et al 1987, Carr et al 1990). However these markers are not necessarily colocalised with each other, for instance SP, SOM and FRAP are only rarely found in the same populations of cells (Hokfelt et al 1976, Dalsgaard et al 1984, Price 1985)

It is probable that the distribution and coexistence of different markers defines subpopulations of neurones, each with their own modalities. However the relationships between the various markers remains to be resolved, making predictions of the modalities of the different subpopulations of neurones difficult.

1.3 Capsaicin

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the active pungent extract from chillies, causes a burning sensation when applied to the skin (Porszasz and Jansco 1959), but renders the excited nerves insensitive to further pain producing stimuli (Jansco and Jansco-Gabor 1959, Jansco 1960, 1968). Systemic administration of capsaicin in the neonatal rat resulted in the loss of 95% of C-fibres (Jansco et al

1977). There is some contradictory evidence that at higher doses there is an effect on Aδ fibres (Wall 1982, Nagy and Hunt 1983) and effects to the central nervous system have also been reported (Fitzgerald 1983, Sivam and Krause 1992). However as a result of the selectivity capsaicin shows for C-fibres capsaicin has been widely used as a pharmacological tool to investigate the role of C-fibres in the development of inflammation and processing of noxious stimuli. However, it should be noted that there is a small population (5%) of C-fibres which are unaffected by capsaicin administration.

Investigation into the effects of systemic capsaicin in adults rats established that dorsal root ganglia were not destroyed, although there was a sensory deficit similar to that seen in neonatal animals (Jansco 1968, Jansco-Gabor et al 1970, Joo et al 1969). It has been suggested that this discrepancy may be due to capsaicin induced destruction of sensory terminals, but not the cells bodies, evidence supporting this theory comes from the observation that systemic capsaicin in the adult rat removes 99% of axons around the ureter, without affecting the number of axons in the innervating dorsal roots (Chung et al 1985). Further research in the same laboratory demonstrated that systemic capsaicin causes the loss of 93% of cutaneous unmyelinated sensory axons, but does not induce the loss of axons in the area of the innervating sural nerve (Chung et al 1990). This suggests that capsaicin-induced neuronal death is limited to the terminal parts of cutaneous sensory axons. However other observers have noted that systemic application of capsaicin in the adult causes a marked loss of small diameter dorsal root ganglion cells, although the dose of capsaicin required to cause this was twice as large (Jansco et al 1985). A consistent feature in adult rats exposed to systemic capsaicin is the depletion of neuropeptides, such as substance P, in central and peripheral terminals of small diameter sensory

neurones (Gamse et al 1980, Jansco et al 1985, Priestley et al 1982, Jessell et al 1978, Lynn 1990). A depletion of neuropeptides in response to capsaicin application has also been reported in DRG cell cultures (Jeftinija et al 1992).

The selectivity of capsaicin in affecting a specific population of neurones has been proposed as evidence for the existence of a specific capsaicin receptor. Recently a receptor, termed VR1, has been cloned from a cDNA library constructed from DRG-derived mRNA (Caterina et al 1997). Upon binding capsaicin, VR1 acts as a highly permeable Ca^{2+} ion channel, fitting with evidence that capsaicin excites nociceptors by increasing the permeability of the plasma membrane to cations (Bevan and Szolcsanyi 1990, Oh et al 1996, Wood et al 1988) and the requirement for extracellular Ca^{2+} ions in electophysiological desensitisation (Holzer 1991, Yeats et al 1992). Furthermore non-neuronal cells transfected with VR1 died upon continuous exposure to capsaicin, consistent with the hypothesis that capsaicin-induced neural death is a result of excessive ion influx (Bevan and Szolcsanyi 1990). Expression of VR1 appeared to be limited to a subset of small diameter DRG neurones, (a much smaller VR1 mRNA transcript was found in the kidney), explaining the selectively of capsaicin for small diameter neurones. Additionally, not all small diameter neurones expressed VR1, which may explain why neonatal capsaicin doesn't destroy all C-fibres. Intriguingly, further evidence from cells transfected with VR1 suggests that a sudden temperature increase causes a Ca^{2+} influx, implicating the receptor as a transducer of painful thermal stimuli *in vivo*.

In the arthritic rats, induced by injected of *M.tuberculosis* around the tibio-tarsal joint, subsequent sub-cutaneous injection of capsaicin caused a decrease in SP and CGRP in DRG and SP in the joint, and caused a decrease in inflammation as measured by joint weight (Ahmed et al 1995 a). Furthermore application of capsaicin to the innervating

nerve of an arthritic joint attenuates the arthritic condition and prevents the spread of arthritis to the contralateral limb in FCA induced bilateral arthritis (Donaldson et al 1995 a). This evidence implicates C-fibres and sensory neuropeptides in the maintenance of experimental peripheral inflammation.

1.4 Neurogenic Inflammation

Neurogenic inflammation is the term coined to describe the involvement of the nervous system in inflammation. The nervous system has been implicated as having a role in inflammation since the observation published by Stricker in 1876 that stimulation of the peripheral end of cut dorsal roots caused mild cutaneous vasodilation, similar to that seen at the site of cutaneous inflammation. Further experiments described the mediation of vasodilation by 'sensory afferent posterior root-fibres', and suggested that this is due to 'impulses passing along sensory fibres in a direction contrary to what is regarded as the usual one'. This phenomenon was termed 'antidromic' (Bayliss 1901, 1923). The observation that neurogenic inflammation in the eye caused by mustard oil application can be abolished by pre-treatment with local anaesthetic or cocaine, or by degeneration of the trigeminal nerve was used as evidence for an axon reflex underlying neurogenic inflammation (Bruce 1910). Inflammatory stimuli cause the triple response, an initial reddening at the site of inflammation is followed by swelling (wheal), and thirdly a reddening of the tissue spreading out from the site of injury (flare). Local anaesthesia of the skin prevented the flare at the site of inflammation, the wheal being unaffected (Lewis 1927), this observation was seen as evidence of a cutaneous network of sensory fibres mediating the flare response. In this arrangement it was postulated that nerve terminals capable of inducing vasodilation were situated around arterioles, distinct from cutaneous

receptors which initiated the axon reflex. (Lewis 1927). However, blocking of action potentials by tetrodotoxin injection did not prevent capsaicin induced plasma extravasation at the site of application (Szolcsanyi 1984). Furthermore, the time course of recovery of human skin from sensory desensitisation caused by capsaicin application was identical for both efferent and sensory functions (Szolcsanyi 1988, 1991). This evidence has led to a revaluation of the axon-reflex theory of neurogenic inflammation. It is now held that neurogenic inflammation is caused by neurochemical release from the same sensory nerve fibres which detect the inflammatory stimulus. As a result neurogenic inflammation can occur at the site of injury without axon reflexes. However axonal conductance is thought to underlie the development of flare away from the site of injection

Vasodilation and plasma extravasation due to release of active substances from peripheral sensory nerves can be caused by stimulation of C-fibres in both the skin (Jansco et al 1967) and cat knee joint (Ferrell and Russell 1986). Neurogenic vasodilation and plasma extravasation can be impaired by prior desensitisation of the skin administration by capsaicin application (Jansco et al 1967). Further experiments have shown that capsaicin-induced impairment of neurogenic inflammation in the skin is mirrored by a decrease of cutaneous SP content, implicating SP in the development of neurogenic inflammation (Gamse et al 1980).

A number of mediators released in response to inflammatory stimuli either stimulate or enhance the excitability of peptidergic sensory nerve fibres. These include interleukin-1β (Fukuoka et al 1994), nitric oxide (Holthusen and Ardnt 1994), prostanoids (Birrell et al 1991), protons (Steen et al 1992), bradykinin (Birrell and McQueen 1993), histamine (Simone et al 1991) and 5HT (Blackshaw and Grundy 1993).

1.5 Neuropeptides in Sensory Nerves

As previously discussed, a number of small peptides are found in sensory nerves, which are implicated as neurotransmitters of sensory information. They are produced in the neuronal cell bodies located within the dorsal root ganglia by transcription of specific genes and translation of their resultant mRNAs., After undergoing postranslational modifications, processed peptides are transported to nerve terminals by axonal transport, and stored in vesicles. Neuropeptides are released in a Ca^{2+} dependent manner upon depolarisation of the nerve terminal, and interact with specific postsynaptic receptors. The neuropeptides of interest in this thesis are members of the tachykinin family and calcitonin gene related peptide, their proposed roles in inflammation and nociception are discussed below.

1.5.1 Tachykinin family

A number of neuropeptides share a common pentapeptide sequence at their carboxy-terminal end, of Phe-X-Gly-Leu-Met-NH_2, and are termed tachykinins. In mammals the tachykinin family of peptides consists of Substance P (SP), Neurokinin A (NKA), two N terminal extensions of NKA, Neuropeptide K and Neuropeptide γ, and Neurokinin B (NKB). The amino acid sequence of SP, NKA and NKB are displayed below (Table 1.2)

Substance P Arg-Pro-Lys-Pro-Gln-Gln-**Phe**-Phe-**Gly-Leu-Met-NH_2**

Neurokinin A His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly-Leu-Met-NH_2**

Neurokinin B Asp-Met-His-Asp-Phe-**Phe**-Val-**Gly-Leu-Met-NH_2**

Table 1.2 Amino acid sequence of members of the tachykinin family of neuropeptides. Amino acids common to all three peptides are shown in bold.

Immunohistochemical studies show SP to be present in cat primary sensory neurones (Hökfelt et al 1975), cell bodies of spinal ganglia (Hökfelt et al 1982) and the dorsal horn of the rat spinal cord (Lembeck and Gamse 1982), implicating SP as a mediator of signalling in primary sensory fibres. Depolarisation of peripheral sensory nerves by electrical stimulation leads to SP release into the dorsal horn of the spinal cord (Yaksh et al 1980). A number of neuropeptides, including SP have been shown to be involved in synaptic transmission in autonomic ganglia (Hökfelt et al 1982) and antidromic vasodilation, an important component of neurogenic inflammation (Olgart et al 1977, Lembeck and Holzer 1979, Hökfelt et al 1982).

The levels of SP and its encoding mRNA, which also encodes NKA (see 1.7), have been shown to be elevated in DRG innervating a rat tibio-tarsal joint injected with FCA (Smith et al 1992, Donaldson et al 1992). In the streptozotocin-induced model of diabetes in the rat there is a decrease in SP in DRG and joint synovium (Garrett et al 1995), reduced conduction velocity in unmyelinated nerves (Zochodne and Ho 1993), decreased axonal transport of SP (Tomlinson et al 1988). Induction of inflammation in the rat knee joint by injection of FCA in the streptozotocin-induced rat model of diabetes produces a reduction in inflammatory response compared to non-diabetic animals (Garrett et al 1996). This evidence implicates both SP and sensory nerves in the development of the inflammatory response to FCA injection.

1.5.2 Tachykinin receptors

Known receptors are termed NK_1, NK_2, NK_3 and NK_4. NK_4 has only been recently identified and little of its pharmacological profile is known (Donaldson et al 1996). The other receptors are well defined and separated based on their varying affinities for SP, NKA and NKB as shown in table 1.3.

	EC_{50} (M)		
	NK_1	NK_2	NK_3
Substance P	3×10^{-9}	8.7×10^{-6}	1.9×10^{-7}
Neurokinin A	5×10^{-8}	6×10^{-8}	2.9×10^{-8}
Neurokinin B	3.6×10^{-7}	3.4×10^{-7}	4.2×10^{-10}

Table 1.3 Affinity of tachykinins receptors for endogenous tachykinins, expressed as concentrations of half maximal effect (EC_{50}) on receptor subtypes expressed in *Xenopus* oocytes (Otsuka and Yoshioka 1993).

Each of the tachykinins will bind to each of the neurokinin receptors, however there is a degree of selectivity amongst the receptors. Thus, substance P is the selective agonist for NK_1, neurokinin A for NK_2, despite having an equal affinity for NK_1 receptors, and neurokinin B the selective agonist for NK_3 receptors.

In the dorsal horn of the spinal cord NK_1 receptors are found primarily in laminae I and II, areas which receives input from SP containing sensory nerves (Yashpal et al 1990). NK_2 receptors are also found in the spinal cord, especially the dorsal and ventromedial borders of the dorsal horn (Yashpal et al 1990). NK_1 and NK_2 receptors are found in a number of sites within the periphery, but particularly near blood vessels. For example, NK_1 receptors are found on perivascular sites in human synovium (Walsh et al 1992). Interestingly NK_1 receptors are reported to increase

under inflammatory conditions, both peripherally at a number of vascular sites, at lymph nodes and in rheumatoid synovium (Mantyh et al 1989, Garrett et al 1998) and centrally in the dorsal horn (McCarson and Krause 1994). NK_3 receptors are only found in spinal cord, especially laminae I and II of the dorsal horn (Yashpal et al 1990). NKB the favoured ligand for NK_3 receptors is also found in the dorsal horn where it is reported to have an antinociceptive effect in the spinal cord (Laneuville et al 1988). NKB is mostly derived from intrinsic spinal neurones (Ogawa et al 1985), and the mRNA for the gene encoding NKB, preprotachykinin B, is absent from the rat sciatic nerve and DRG (Moussaoui et al 1992).

1.5.3 Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide, two forms exist in both humans and rats; α-CGRP, produced by alternative splicing of calcitonin gene mRNA in neuronal tissue (as shown in fig 1.1) (Amara et al 1982), and β-CGRP, the sole product of a separate gene (Amara et al 1985). These two peptides are very similar, differing by 3 amino acids in humans and 1 in rat.

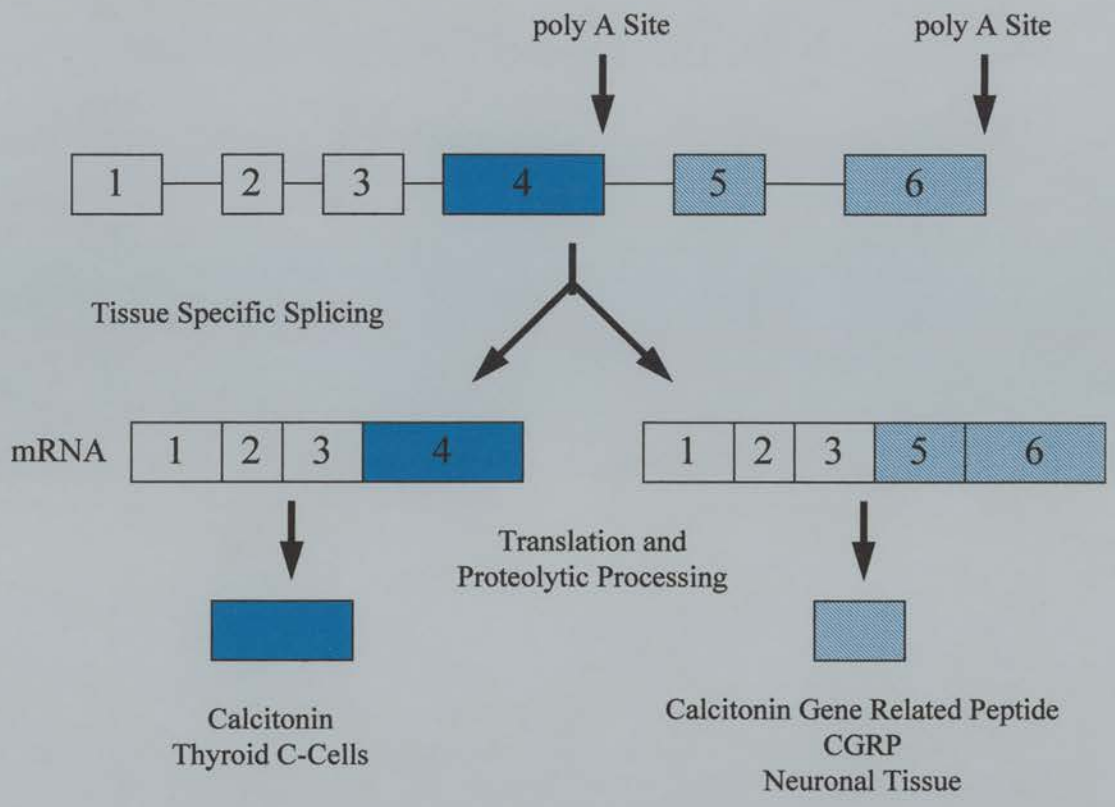


Figure 1.1 Calcitonin and calcitonin gene-related peptide (CGRP) are produced by tissue specific alternative splicing of the same gene. Adapted from Amara et al 1982

CGRP is widely distributed throughout the central and peripheral nervous systems, in both motor and sensory nerves, where the α-CGRP form is present. In rat sensory nerves, CGRP has been shown to be colocalised with a number of other neurotransmitters, including VIP, SOM, neuropeptide Y and especially SP (Ju et al 1987). This co-localisation suggests that CGRP rarely works on its own, and a role as a neuromodulator has been suggested (Poyner 1992). Experiments involving application of CGRP to myenteric neurones of guinea pig ileum showed that CGRP induced the closure of potassium channels (Palmer et al 1986), causing a prolonged increased excitability of the neurones. This is the mode of action of an enhancer of a second depolarising stimulus, and indeed CGRP has been shown to act as an enhancer in the rat dorsal horn, where prior administration of CGRP potentiated the responses

of substance P and noxious stimulation (Biella et al 1991). Additionally, intrathecal CGRP increases the duration of the substance P-induced caudally directed biting and scratching in rats and mice, without having any effect when administered alone (Wiesenfeld-Hallin et al 1984). These two peptides have been shown to act together in lowering the threshold of the nociceptive flexor withdrawal reflex in rats (Woolf and Wiesenfeld-Hallin 1986). In addition CGRP can potentiate the release of substance P from the dorsal horn neurones of the rat, suggesting a interaction with a presynaptic receptor (Oku et al 1987) and can inhibit substance P degradation centrally and peripherally (Le Greves et al 1985, Mao et al 1992).

1.5.4 CGRP receptors

Lack of availability of selective non-peptide antagonists has clouded research into CGRP pharmacology. CGRP receptors are classified as $CGRP_1$ and $CGRP_2$ depending upon the different biological activity of CGRP analogues, especially CGRP 8-37, which shows specificity for $CGRP_1$ receptors (Mimeault et al 1991). Based on the selectivity for CGRP 8-37, the $CGRP_1$ receptor subtype are proposed to be present in the dorsal horn of the spinal cord (Lofgren et al 1997). A third receptor subtype is proposed in the nucleus accumbens (Van Rossum et al 1997).

1.6 Action of SP, NKA and CGRP in Sensory Nerves

During acute peripheral inflammation induced by injection of *M. butyricum* there is an increase in density of neurones containing and releasing CGRP (Hanesch et al 1994) as well as an increases in CGRP in innervating DRG (Kuraishi et al 1989). SP and its encoding mRNA are upregulated in DRG innervating joints inflamed by FCA injection, a stimulus which also increases α-CGRP encoding mRNAs (Smith et al 1992, Donaldson et al 1992). The increase in mRNAs and peptide levels in DRG is mirrored in increases in peptide levels released from sensory nerve terminals. Immunoreactivity of SP, NKA and CGRP increases in the synovium of the knee joint of rats with acute adjuvant induced monoarthritis (Bileviciute et al 1993).

There is also an increase in levels of SP in the dorsal horn of the spinal cord in FCA induced polyarthritic rats (Oku et al 1987). CGRP release into the dorsal horn is increased in FCA-induced polyarthritis (Collin et al 1993). SP and NKA has been shown to be increased centrally in response to cat acute arthritis induced by kaolin/carrageenan injection (Schaible et al 1990, Hope et al 1990).

These data implicate sensory neuropeptides in the development of the inflammation, both centrally and peripherally.

1.6.1 Peripheral actions of tachykinins and CGRP

An overview of the events underlying inflammation is described below (Fassbender 1994, Rang and Dale 1981). The first event in the inflammatory process is the detection of damage, mediators released in response to this damage effect the local capillary network and post capillary venules. Vasodilation of these blood vessels causes increased blood flow to the inflamed area, which is evident in reddening of the site of inflammation. The increased blood flow increases transport of components of both the humoral (e.g. complement system) and cellular (e.g. neutrophils) defence mechanisms, to the site of inflammation. Inflammatory mediators also increase vascular permeability, allowing humoral factors to enter the inflamed tissue. Plasma enters the tissue, via the enlarged gaps between the endothelial cells lining the capillaries. The alteration in osmotic balance by the movement of plasma causes inflow of water into the inflamed tissue, resulting in swelling (oedema). Inflammatory cells also move from the blood vessels to the surrounding tissue, this movement is regulated by the binding between cell surface ligands (adhesion molecules) expressed by the endothelial cells lining blood vessels and cell surface receptors (intergrins) expressed by inflammatory cells. Expression of adhesion molecules is regulated by inflammatory mediators such as cytokines. Inflammatory cells actively migrate to the site of inflammation in response to chemicals release (chemotactic stimuli). The first cells to infiltrate the inflamed area are the granulocytes, including polymorphonuclear leukocytes, followed by monocytes, macrophages and lymphocytes. Once at the site of inflammation the inflammatory cells neutralise or remove the trigger of inflammation.

Some of the interactions between immune cells, microvascular vessels and peripheral neurones are outlined in figure 1.2. Additional events in the inflammatory process involving SP, NKA and CGRP are discussed below.

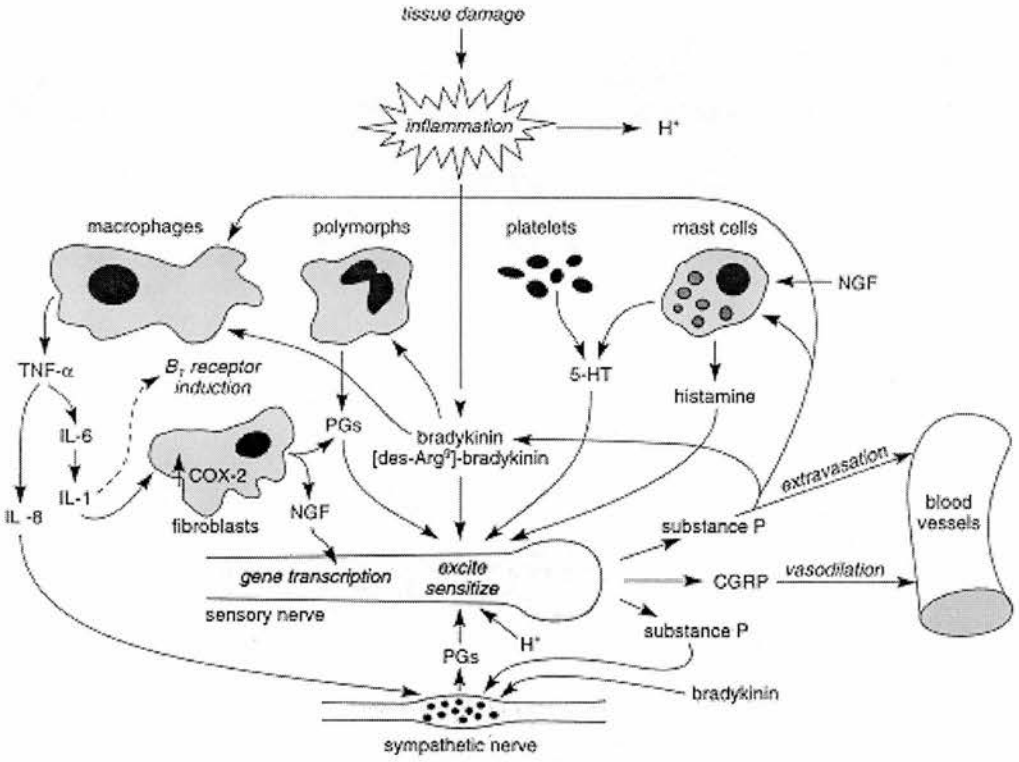


Figure 1.2 Summary of some of the interactions between the immune system, nervous system and microvessels during inflammation. CGRP, calcitonin gene-related peptide; COX-2, cyclooxygenase enzyme 2; 5-HT, 5-hydroxytryptamine (serotonin); IL-1, IL-6 and IL-8 interleukin 1, 6 and 8 respectively; NGF, nerve growth factor; PGs, prostaglandins; TNF-α, tumour necrosis factor α. B_1 receptors refer to bradykinin type 1 receptors. Modified from Dray and Bevan (1993).

1.6.1.1 Vascular effects of the tachykinins and CGRP

Release of tachykinins and CGRP cause increased blood flow in the rat knee joint (Lam and Ferrell 1993), implicating these peptides in vasodilation of joint microvasculature. Comparison of the vasodilator activity of these neuropeptides revealed CGRP to be the most potent vasodilator, suggesting it to be the primary mediator of vasodilation in neurogenic inflammation. The CGRP mediated

vasodilation in skin is postulated to be as result of CGRP interaction with $CGRP_1$ receptors (Brain 1996). Increased vascular permeability in the rat knee joint has been shown to be primarily mediated by the action of SP at NK_1 receptors (Lam and Ferrell 1991, Hirayama et al 1993).

1.6.1.2 Neuropeptides in the development of primary hyperalgesia

Primary hyperalgesia occurs at the site of inflammation, it is due either to direct activation of peripheral endings of sensory afferents in the region of inflammation, or an indirect activation caused by the sensitisation of sensory nerve terminals. The latter occurs during kaolin/carrageenan-induced inflammation in the cat knee joint (Guilbaud et al 1985, Schaible and Schmidt 1985). As discussed in 1.4, activation or sensitisation of peripheral sensory nerve terminals can be caused by a number of inflammatory mediators, but is not thought to be a direct result of tachykinin or CGRP action. However these neuropeptides can augment the inflammation, for instance SP causes release of histamine from mast cells (Krumins and Broomfield 1993), which then sensitises sensory nerves.

1.6.1.3 Interactions between the immune system and sensory neuropeptides

Inflammatory joint conditions, such as rheumatoid arthritis (Fassbender 1994) and FCA induced inflammation (Donaldson et al 1993) are associated with infiltration of inflamed tissue by cells of the immune system. SP, NKA and CGRP have been implicated in modulation of inflammatory cell chemotaxis and function.

Neutrophils

This class of polymorphonuclear leukocytes (PMN) actively ingest invading pathogens by phagocytosis and kills them by the action of oxygen metabolites such as superoxide radicals (O_2^-) and hydrogen peroxide. Lytic enzymes contained within intracellular lysosomes then digest the pathogen. SP and CGRP are implicated in

modulating the initial adherence of neutrophils to the endothelial lining of microvessels as both neuropeptides cause neutrophil adherence to monolayers of human endothelial cells (Zimmerman et al 1992). It also appears that SP has a chemotactic effect on neutrophils, causing the accumulation of these immune cells. The accumulation of neutrophils has been shown to be mediated by two different mechanisms, either by NK_1 receptors in guinea-pig (Walsh et al 1995), or via the activation of mast-cell in rat (Suzuki et al 1995), the latter pathway also causes the accumulation of other leukocytes. SP also potentiates the production of superoxide by human neutrophils (Serra et al 1994).

CGRP may also have a chemotactic effect, and is suggested to mediate the accumulation of mouse neutrophils in response to IL-1 (Ahluwalia et al 1994). Another potential role for CGRP is the activation of neutrophils, which has been demonstrated in human neutrophils (Richter et al 1992).

Lymphocytes

Lymphocytes are the class of white blood cell which includes B and T cells which mediate antigen-specific immune reactions. Both B and T cells have been implicated in the aetiology of both rheumatoid arthritis and arthritic conditions induced by FCA injection (Rainsford 1982). Experimental evidence suggests that SP causes proliferation of human lymphocytes (Payan et al 1983). Conversely, CGRP appears to have a down-regulatory role, inhibiting the following; mitogen-stimulated proliferation of murine T-lymphocytes (Umeda et al 1988), production of interleukin 2 in mouse T lymphocytes (Wang et al 1992) and differentiation in the murine 70Z/3 pre-B lymphocyte cell line (McGillis et al 1993).

Other inflammatory cells

Experimental evidence suggests that SP and CGRP have effects on other

inflammatory cells. SP causes the production of the inflammatory cytokines, interleukins 1β, 6 and 10 and tumour necrosis factor-α, in primed human monocytes (Lieb et al 1996, Laurenzi et al 1990, Ho et al 1996) and the release of histamine from mast cells (Krumins and Broomfield 1993). It has been also suggested that SP may play a role in priming mast cells in rat (Janiszewski et al 1994). CGRP enhances phagocytosis in cultured mouse macrophages (Ichinose and Sawada 1996), but inhibits both the production of hydrogen peroxide and the ability of human macrophages to present antigen (Nong et al 1989)

1.6.1.4 The role of the tachykinins and CGRP in tissue repair and wound healing

On removal or neutralisation of the trigger of inflammation, the inflammatory reactions cease and repair processes take over. It has been suggested that neuropeptides may act as cellular growth factors, playing a role in the tissue repair and wound healing (Rosengurt 1991). SP has reported proliferative effects upon human skin fibroblasts (Ziche et al 1990 a) and synoviocytes (Lotz et al 1987) and rat smooth muscle cells (Payan 1985). There is evidence that SP and CGRP can exert proliferative effects on human endothelial cells (Ziche et al 1990 b, Haegerstrand et al 1990) and that SP can induce the mobilisation of bovine capillary endothelium *in vitro* (Ziche et al 1991). These observations suggested a role for SP in angiogenesis, and this has been observed *in vivo* in the rabbit cornea (Ziche et al 1990 a). Furthermore the survival of critical skin flaps in the rat is increased by application of tachykinins and CGRP, implying these neuropeptides may have a role in wound healing (Kjartansson et al 1987).

1.6.2 Central action of tachykinins and CGRP

Terminals immunoreactive for SP are found in laminae I, II, V and X of the spinal dorsal horn, areas where primary afferent nociceptive fibres terminate (Gibson et al 1981), and also in the laminae (I and II) with the highest density of NK_1 receptors (see section 1.5.2). SP is released into the spinal cord in response to a variety of stimuli associated with nociception. These stimuli include excitation of C-fibres by electrical stimulation (Yaksh et al 1980) or capsaicin application (Zhao et al 1992), and noxious thermal, mechanical or chemical stimulation of the skin (Duggan et al 1988). Thermal and mechanical stimulation of the hind paw at intensities above nociceptive thresholds increases NKA and CGRP in the dorsal horn (Duggan et al 1987, Morton and Hutchinson 1989). CGRP is also released into the dorsal horn upon electrical stimulation of the sciatic nerve at intensities that activate C fibres (Klein et al 1990) and in response application of the inflammatory mediators, bradykinin and prostaglandins to the rat spinal cord (Andreeva and Rang 1993). These experimental data suggest that SP, NKA and CGRP may be involved in the central response to nociceptive input. Studies employing the specific NK_1 antagonist, CP 99,994, and NK_2 antagonist SR 48968 have provided evidence that both and NK_1 and NK_2 receptors are involved in central transmission of nociception (Neugebauer et al 1996, Sluka et al 1997). It has been suggested that NK_1 and NK_2 receptors play different roles in processing nociceptive information, for example it is appears that NK_2 receptors are involved in the induction of heat hyperalgesia and NK_1 receptors in the maintenance of heat hyperalgesia (Sluka et al 1997). Additionally intrathecal administration of the $CGRP_1$ receptor antagonist, CGRP 8-37, results in decreased nociception in thermally injured rats (Lofgren et al 1997).

Secondary hyperalgesia describes the state when hyperalgesia is observed at a site

away from the initial inflammatory stimulus. For example, there is a decrease in paw withdrawal latency in response to thermal stimuli within 4h and up to 24h after kaolin/carrageenan injection into the rat knee joint (Sluka and Westlund 1993). It has been suggested that there is a central contribution to secondary hyperalgesia observed after hindpaw injection of carrageenan in rats (Guilbaud et al 1992). There is also a reported enhanced excitability in spinal cord neurones in the kaolin/carrageenan model of joint inflammation (Neugebauer and Schaible 1988) and adjuvant-induced hindlimb inflammation (Hylden et al 1989), suggesting that the development of spinal excitability may underlie secondary hyperalgesia. The role of SP, NKA and CGRP in increased dorsal horn excitability is discussed below.

Activation of either low threshold Aβ fibres or high threshold Aδ and C fibres results in increased aspartate and glutamate in the spinal cord (Kangrga and Randic 1991). In non-inflamed conditions glutamate released from Aβ fibres, activates AMPA receptors, giving short lasting excitations (Dougherty et al 1992). Under non-inflamed conditions glutamate binding at NMDA receptors has little effect due to blockage of the ion channel in the by a Mg^{2+} ion, preventing glutamate induced Ca^{2+} influx. The ion channel block is lifted in response to repeated C-fibre input into the rat dorsal horn, resulting in NMDA receptor activation (Dickenson and Sullivan 1987, 1990). As previously discussed neuropeptides are found in C-fibres, these include SP, 90% of SP containing fibres also contain glutamate (Battaglia and Rustioni 1988). SP, as well as NKA and CGRP, are reported to facilitate the effects of glutamate at NMDA receptors in the slices of rat dorsal horn (Randic et al 1990, Kangrga and Randic 1990). It is proposed that the action of these neuropeptides on receptors in laminae I and II sensitise the dorsal horn to input from the periphery (Woolf and Doubell 1994). Stimulation of neuropeptide-containing small diameter afferents causes a slow post-

synaptic potential in cat dorsal horn neurones, an effect which can be mimicked by ionophoretic application of SP (Randic and Miletic 1977). On repeated stimulation of small diameter rat afferents, slow post-synaptic potentials summate and cause depolarisation of the postsynaptic membrane of the dorsal horn (Sivilotti et al 1993). The depolarisation of the dorsal horn leads to a state of hyperexcitability and an increased sensitivity to peripheral input, including low intensity inputs (Thompson et al 1993). The hyperexcitability of the dorsal horn, also termed wind-up, can be prevented by prior ionophoretic administration of the selective NK_1 receptor antagonist, CP 96,345, suggesting SP may be involved in the aetiology of wind-up (Neugebauer et al 1995). The cellular mechanisms underlying wind up are summarised in figure 1.3

Wind-up results in increased sensitivity of the dorsal horn to the release of EAA from both C and Aβ fibres. This produces exaggerated responses to painful stimuli (secondary hyperalgesia) and also the perception of previously innocuous stimuli as painful. Dorsal horn sensitisation is reinforced by a phenotypic switch in a population of Aβ-fibres, which start expressing substance P (Neumann et al 1996).

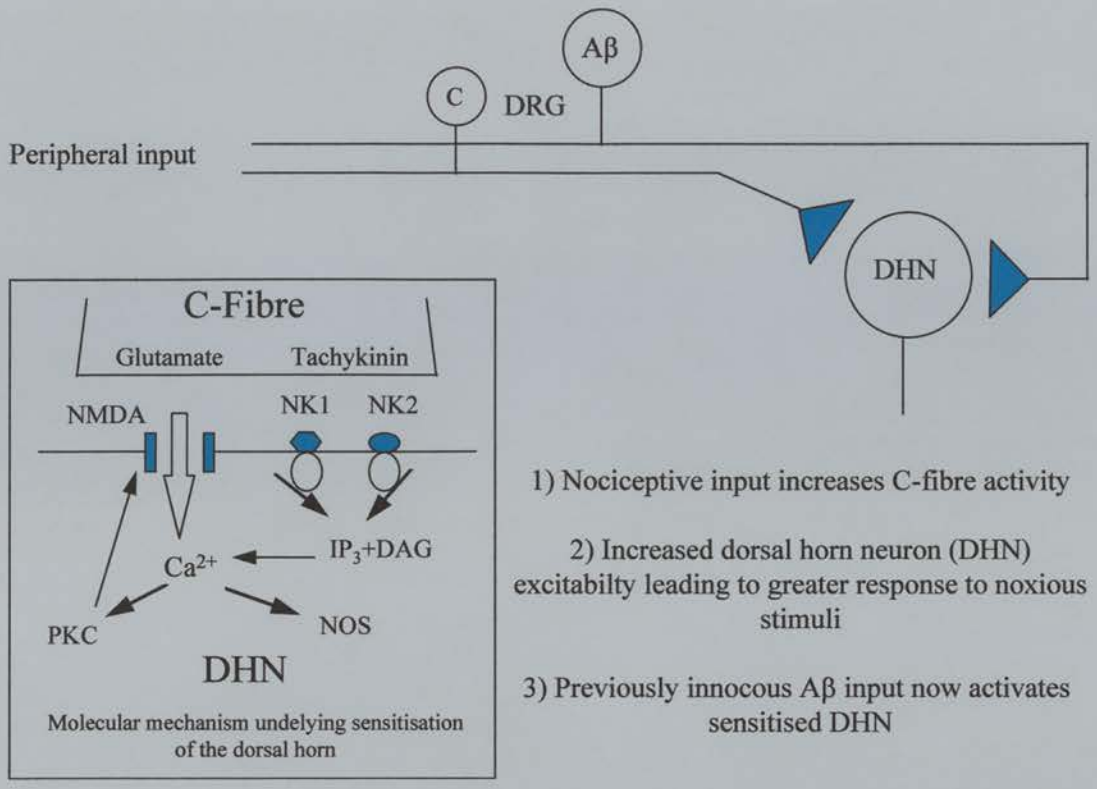


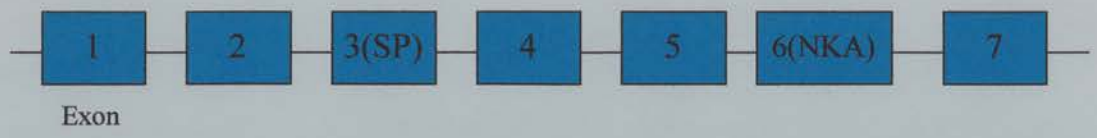
Figure 1.3 Cellular mechanism underlying central sensitisation and wind up. SP and NKA released from C-fibres binds to specific receptors in the dorsal horn. SP and NKA binding activates the IP_3 signalling pathway (Nakajima et al 1992). The resulting increased Ca^{2+} mobilisation causes protein kinase C (PKC) activation. The NMDA receptor is phosphorylated by PKC, relieving Mg^{2+} block of the NMDA ion channel. The lifting of the ion channel block sensitises the dorsal horn to further release of glutamate and other excitatory amino acids, both from Aβ and C fibres. Adapted from Woolf and Doubell 1994

The Ca^{2+} influx into the dorsal horn caused by glutamate binding to NMDA receptors leads to an activation of cellular targets within the spinal cord, notably the induction of the immediate early genes c-fos (Noguchi et al 1991), c-jun and NGFI-A (Herdegen et al 1991) and activation of nitric oxide synthase (NOS). Nitric oxide, produced by NOS, has been suggested in having a pro-inflammatory role in spinal processing (Meller and Gebhart 1993).

Experimental evidence presented in section 1.6 implicates the release of tachykinins and CGRP from sensory nerves in multiple processes underlying inflammation. Therefore, the control of the expression of genes encoding these neuropeptides is of considerable interest and relevance to the aetiology of events in FCA-induced inflammation.

1.7 Tachykinin Gene Expression

The levels of the tachykinins are dependent upon the expression of their encoding gene. SP, NKA, and the related Neuropeptide K and Neuropeptide γ, are encoded by the gene preprotachykinin A (PPT-A). Alternative splicing of a common pre-mRNA results in 4 different PPT-A mRNA transcripts, termed α,β,γ and δ as displayed in figure 1.4 This leads to discrete expression of various peptide combinations in different tissues. In DRG neurones mainly the β and γ forms are present, as a result SP and NKA are expressed in DRG neurones. Neurokinin B is encoded by a separate gene, PPT-B, which is not expressed in DRG (Moussaoui et al 1992).



Alternative Splicing Variants of the PPT-A Gene

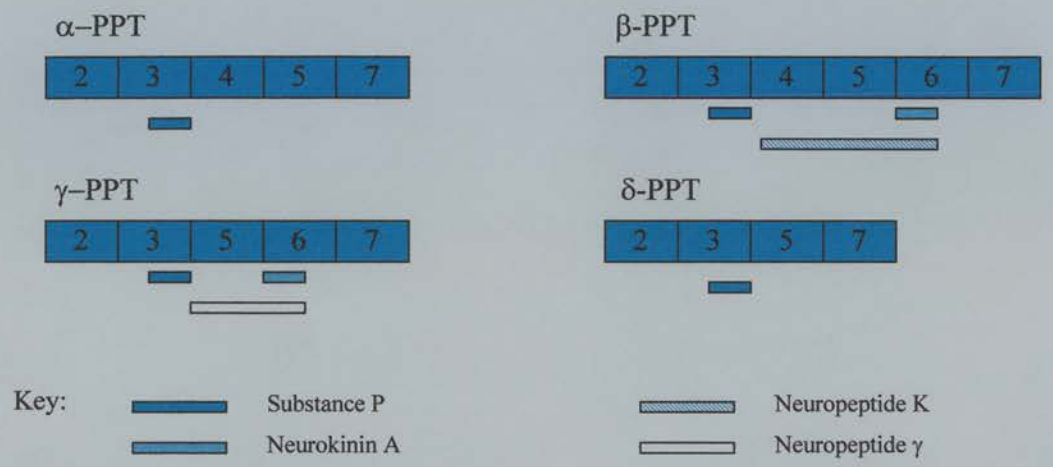


Figure 1.4 Alternative splicing of PPT-A gene, which encodes substance P, neurokinin A, neuropeptides K and γ, leads to different splicing variants

Translation of the mRNAs leads to production of large precursor molecules. These contain a signal peptide, directing the peptide to the correct cell compartment, the individual tachykinins (separated by spacer sequences) and a carboxy terminal sequence. The tachykinins are cleaved out of the precursor molecules by specific enzymes, termed convertases, which typically cleave at doublets of basic amino acids. The cleaved peptide then undergoes carboxy-terminal amidation, a step that is crucial for substance P inflammatory action (Ogonowski et al 1997).

1.8 Regulation of PPT-A Expression

PPT-A expression will have a direct effect on the levels of SP available for transmission of neural information. The mRNA levels of PPT-A increase in DRG innervating a joint inflamed by injection of carrageenan (Iadarola and Draisci 1988),

formalin (Noguchi et al 1988), or FCA (Donaldson et al 1993, Heppelmann et al 1993). Experiments carried out in cultures of sympathetic and sensory neurones have shown that SP expression can be regulated by neurotrophic growth factors (Lindsay and Harmar 1989), conditioned media (Nawa and Sah 1990, Nawa and Patterson 1990) and cytokines (Freiden and Kessler 1991, Shadiack et al 1993, Fann and Patterson 1994). *In vivo* expression can be regulated in the striatum by cocaine (Hurd et al 1992), and the hippocampus by depolarisation with kainate (Brene et al 1992), whilst levels of SP in DRG are affected by glucocorticoids, (Smith et al 1991). It has been suggested that these stimuli may act at the level of transcription (Paterson et al 1995 a).

The region 3.3kb 5' to the start point of transcription of the rat PPT-A gene is capable of directing reporter gene expression in adult rat DRG neurones grown in culture (Harmar et al 1993). Deletion analysis of this 3.3kb sequence has implied the presence of two enhancer regions between -755 to -565 and +92 to +527 (the transcriptional start point is designated as +1) (Mulderry et al 1993 a). Analysis of the region 865bp 5' of the transcriptional start point has revealed a number of putative transcription factor binding sites, including cAMP response elements (CRE) and AP1 and octamer binding sites (Carter and Krause 1990, Chapman et al 1993).

As discussed in section 1.2, DRG have a heterogeneous cell population, making the obtainment of a homogenous nuclear extract for biochemical analysis difficult. The non-neuronal HeLa cell line expresses a large array of transcription factors also present in DRG neurones, including members of the octamer, AP-1(jun/fos), bHLH (E box) and cAMP responsive element binding protein (CREB) families (Fiskerstrand and Quinn 1996).

The region spanning -865 to + 500 has been studied using HeLa nuclear extracts and

the techniques of DNAse 1 footprinting and electromobility shift assay (EMSA) in order to identify the transcription factors which interact with this region *in vitro*. Located in this region of the rat PPT-A gene are sites which interact with AP-1/CREB (Paterson et al 1995a, Morrison et al 1995), octamer binding protein (Mendelson et al 1998), members of the bHLH (E box) family (Mendelson and Quinn 1995, Paterson et al 1995 a,b and c) and the Sp 1 family (Quinn et al 1995, Mendelson et al 1998). Interestingly within the enhancer regions active binding sites for octamer binding proteins and members of the Sp 1 and NFκB families have been identified (Quinn et al 1995, Fiskerstrand et al 1997, Mendelson et al 1998).

1.9 CGRP Expression

The control of CGRP gene expression has not been widely studied in neuronal cells, however it has been shown that an enhancer element defined in thyroid cells spanning the region -1127 to -957 can also stimulate promoter activity in neuroblastoma cell lines (Stolarsky-Freedman et al 1990). NGF has been shown to increase CGRP and its encoding mRNA up to 15 fold in cultured dorsal root ganglia, which were not dependent upon NGF for survival (Lindsay 1988, Lindsay and Harmar 1989). Levels of mRNAs encoding CGRP in cultures of adult DRG are also increased by activators of protein kinase A and protein kinase C (Supowit et al 1995). Subsequent studies have determined enhancer regions necessary for the NGF-induced induction of a minimal CGRP promoter transfected into cultures of adult DRG (Watson et al 1995) and cAMP response element in the promoter region (Watson and Latchman 1995). The NGF induced increase in CGRP mRNA observed in cultures of adult DRG can be prevented application by the synthetic glucocorticoid, dexamethasone (Supowit et al 1995). CGRP mRNA has also been reported to be increased in neuronal cells by the

application of activin A, a member of the transforming growth factor β superfamily of cytokines (Fann and Patterson 1994).

1.10 Messenger RNA and its Quantification

The expression of specific genes can be investigated by quantifying the amount of mRNA produced. The sequence of DNA is copied, or transcribed by RNA polymerase enzymes in the cell nucleus. The rate of transcription is either facilitated or inhibited by modulatory proteins known as transcription factors, which bind to specific sequences of nucleotides within promoter regions of genes. The direct copy of the DNA sequence containing sequences encoding protein (exons) separated by non-coding regions (introns) is termed heterogeneous nuclear RNA (hnRNA). hnRNA is a very short lived species the introns are rapidly spliced out and degraded, leaving a mature mRNA containing only exons. Mature, spliced mRNA moves to the cytoplasm where it can be translated at ribosomes into the encoded peptide.

mRNA (or hnRNA) can be quantified using in-situ hybridisation, which utilises a synthesised oligonucleotide probe, complementary to the sequence of the mRNA of interest. This complementary probe binds to the RNA species, and can be visualised and quantified by a label, such as radioactive ^{35}S-UTP. The advantage of in-situ hybridisation over other techniques for quantifying mRNA is that synthesis can be studied in specific locations, such as different cell sub-populations. In DRG there is a heterogeneous cell population, the classification of which is described in section 1.2.

1.11 Transcription Factors in DRG

The activational state of transcription factors in DRG and their ability to bind to the promoter region of PPT-A and the α-CGRP encoding gene are important in understanding the upregulation of these neuropeptide encoding genes which appear to underlie the aetiology of FCA induced inflammation.

Transcription factors can be broadly divided into 2 groups, those that are made *de-novo* in response to a stimulus, for example the immediate early genes, and those present in the cell which are activated by a post translational event such as phosphorylation (reviewed by Jans 1995).

Previous analysis of the expression of mRNAs encoding transcription factors in DRG has shown that members of the AP-1 family of transcription factors, NGFI-A and NGFI-B are either not present in DRG or their levels are unaltered in the first 8h after FCA injection (Donaldson et al 1995 b). The AP-1 members, c-jun and junD have been shown to be upregulated in DRG after sciatic nerve axotomy (Kenney and Kocsis 1997), a condition characterised by a decrease in the expression of PPT-A and α-CGRP encoding mRNAs (Verge et al 1995). Levels of mRNA encoding the transcription factor AP-2 increase in DRG innervating an FCA inflamed tibio-tarsal joint within 1h of FCA injection (Donaldson et al 1995 b). Intriguingly EMSA analysis has revealed an active AP-2 site in the promoter region of the rat PPT-A gene between bases -865 and -47 (Quinn et al 1995), suggesting that AP-2 could regulate PPT-A expression.

The involvement of those transcription factors made *de novo*, such as AP-2, in the upregulation of neuropeptides following FCA induced inflammation, can be assessed by inhibiting protein synthesis prior to FCA injection. Cycloheximide is a general inhibitor of eukaryotic protein synthesis, inhibiting peptidyl transferase, the enzyme

responsible for forming the peptide bond as detailed in figure 1.5. Given that cycloheximide is a potent inhibitor of protein synthesis, and the vital role proteins occupy in cell processes and metabolism necessary for cell survival, high doses of cycloheximide are lethal. However work carried out studying the *in vivo* incorporation of amino acid in rat liver and kidney have demonstrated that it is possible to inhibit protein synthesis with a small, sub-lethal dose of cycloheximide (Rothblum et al 1976). A dosage of 3mg/kg administered systemically causes a 85% decrease in the incorporation of [^{3}H] leucine within 1h of intraperitoneal injection. This inhibition is reversible, with incorporation returning to untreated levels at 48h.

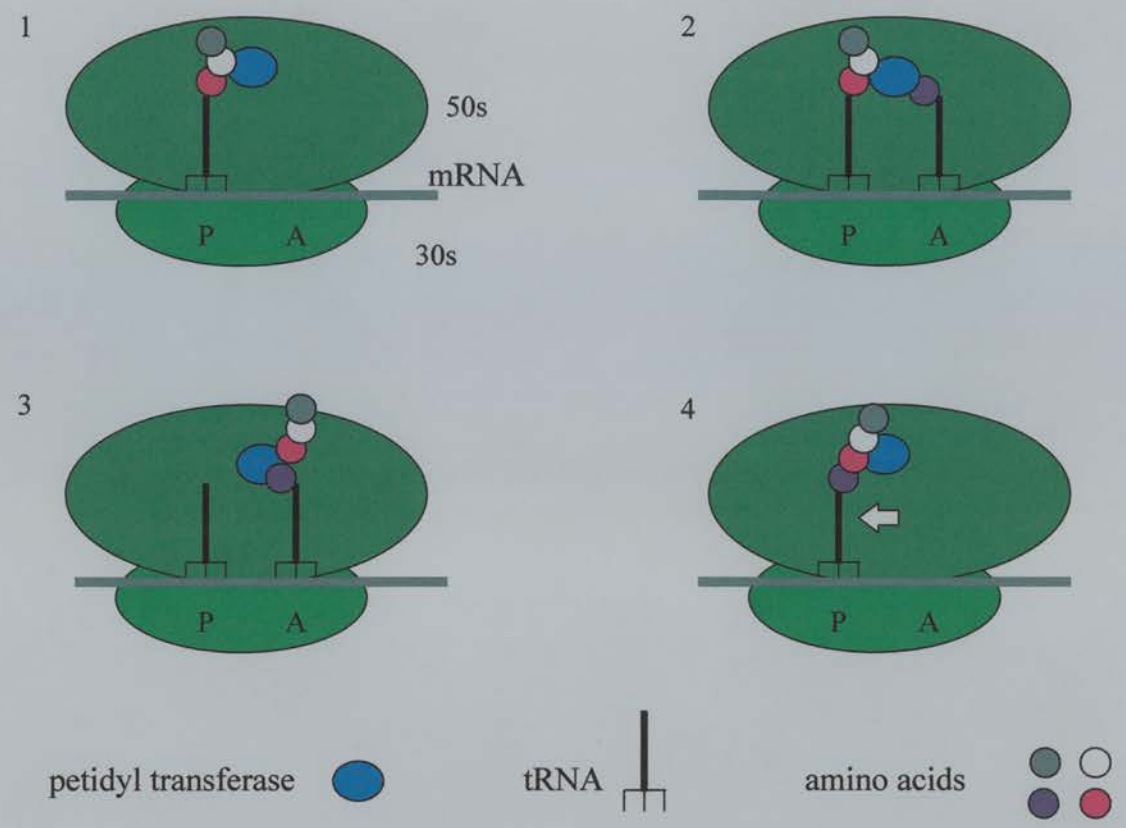


Figure 1.5 Diagram of elongation phase of protein synthesis in eukaryotes

1) Growing polypeptide chain attached by the terminal aminoacyl -tRNA group to the P site of the ribosome. A site empty.

2) Binding of specific aminoacyl-tRNA molecule by hydrogen bonding at the A site of the ribosome.

3) Peptide bond formed between the amino acid chain and the amino acid in the P site. This is mediated by the action of peptidyl transferase.

4) The tRNA in the P site is ejected and the growing polypeptide chain translocates from the A site to the now empty P site Thus the ribosome moves along the mRNA, and the next amino-acyl tRNA complementary to the mRNA sequence can enter at the empty A site (step 2)

Cycloheximide acts by inhibiting the actions of peptidyl transferase, preventing the growth of the polypeptide chain and arresting protein synthesis. The mode of action is non-selective, effecting all ribosomes, thus cycloheximide is a general inhibitor of protein synthesis.

Adapted from Watson et al 1987

1.12 Neurotrophins and Neuropeptide Expression

Nerve growth factor (NGF), a member of the neurotrophin family, has been shown to be essential for survival of sympathetic and sensory neurones during development (Thoenen and Barde 1980), and subsequently shown to positively regulate β-PPT A and α-CGRP encoding mRNAs in cultures of adult dorsal root ganglia grown in the absence of neuronal growth factors (Lindsay and Harmar, 1989). The identification of NGF responsive elements in the promoter region of both PPT-A (Gilchrist et al 1991) and the α-CGRP encoding gene (Watson et al 1995) and observations of the ability of NGF to regulate neuropeptides genes in DRG in an *in vivo* situation (Amann et al 1996, Vedder et al 1993) have lead to a hypothesis that NGF is a mediator of plastic changes in sensory neuropeptides during inflammation. Levels of NGF, produced in smooth muscle cells, basal keratinocytes and fibroblasts, increases in the sciatic nerve and peripheral tissue during experimentally (FCA) induced inflammation (Donnerer et al 1992, Safieh-Garabedian et al 1995). It has been proposed that NGF transported from the periphery to the cell body of the sensory nerve by retrograde axonal transport causes an up-regulation in neuropeptide gene expression (Amman et al 1996). Indeed in rats with transected peripheral nerves, a situation which would abolish retrograde axonal transport, including that of NGF from the periphery to DRG, there is a decrease in the synthesis of SP and CGRP, which can be reversed by NGF administration (Verge et al 1995). The rate of retrograde NGF transport in the rat sciatic nerve has been measured at 13 mm/h (Stockel et al 1975). As a result it would take several hour between the production of NGF in the periphery and effects of NGF on neuropeptide gene expression in DRG.

Other members of the neurotrophin family include brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4). The latter is also known

as NT-4/5 or NT-5. BDNF has been reported to induce preprotachykinin expression in the central nervous system (Croll et al 1994, Nawa et al 1994). However neither BDNF or the related neurotrophin 3 (NT-3) induce PPT-A expression in adult DRG neurones, both *in-vitro* (Mulderry 1994) and *in-vivo* (Zhang et al 1995).

Using in situ hybridisation PPT-A and α-CGRP have been shown to be co-localised in small diameter (≤ 25μm) nerve fibres with the high affinity NGF receptor, trkA (Kashiba et al 1996). The high affinity receptors for BDNF (trkB, which also bind NT-4) and NT-3 (trkC) are found in DRG neurones, but are not co-expressed with the mRNA encoding PPT-A, although a very small number of neurones expressed both trkC and α-CGRP transcripts (Kashiba et al 1996). It should be noted that this study demonstrated that not all neurones positive for PPT-A or α-CGRP mRNA also expressed high affinity neurotrophin receptor mRNAs. These neurones include a subpopulation of small diameter cells and all large diameter cells expressing PPT-A mRNA. Additionally not all trkA mRNA positive cells also expressed PPT-A or α-CGRP encoding mRNAs.

The low affinity neurotrophin receptor, p75, which binds all members of the neurotrophin family, is also expressed in DRG neurones, in fibres of all diameters. Expression of p75 is limited to cells expressing one of the trk high affinity neurotrophin receptor subtypes (Wright and Snider 1995), fitting with p75's proposed role of regulating trk signalling (Verdi et al 1994). However, recent experimental evidence that binding of NGF to p75 causes the activation of the transcription factor NFκB in Schwann cells (Carter et al 1996) suggests that the p75 receptor may play a role in signalling independently of high affinity trk receptors. The precise relationship between p75 and PPT-A/CGRP expressing DRG neurones has not been studied.

1.13 Gene Knockout Studies

Recently mutant mice have been generated with targeted deletion of the SP and NKA coding regions of PPT-A (Cao et al 1998) or the gene encoding NK_1 receptors (De Felipe et al 1998), with the aim of elucidating the role of SP and NKA in inflammation and pain transmission.

1.13.1 PPT-A knockout

Mice with a disrupted PPT-A gene show similar responses to mildly painful stimuli as wild type mice, but the responses to moderate to intense thermal, mechanical or chemical pain are significantly decreased. However at very high intensities of pain, wild type and homozygous knock-out mice displayed similar responses, thus suggesting that SP and NKA are involved in the transmission of painful stimuli within a specific range or window. Capsaicin specifically activates C fibres (see section 1.3), greatly decreased plasma extravasation and oedema formation in response to capsaicin in homozygous mutants compared to wild type mice, implicates SP release from C fibres as pivotal to these responses. However this hypothesis is complicated by the fact that heterozygous animals also display decreased plasma extravasation and oedema in response to capsaicin. FCA injection causes development of oedema and allodynia (decreased nociceptive threshold) to a similar degree in both wild type and mutant mice, suggesting that these events are not solely dependent on SP. As depicted in figure 1.2 other substances produced in response to inflammation have been implicated in the development of inflammatory processes.

1.13.2 NK1 knockout

The NK_1 receptor shows highest affinity for substance P, mutant mice lacking this receptor sub-type did not display a graded response to increasing electrical and

mechanical stimulation, nor did they display 'wind up'. Mutant mice also showed decreased neurogenic inflammation in response to capsaicin and carrageenan, although after FCA injection mutant mice displayed oedema, erythrema and mechanical hyperalgesia similar to that seen in wild type mice.

1.13.3 Implication of roles of SP and NKA in inflammation

The similarity of behaviour and inflammatory responses in both mutant animals suggests that neither SP or NKA is the primary transmitter of painful stimuli, with glutamate being the most likely candidate for this role. SP and NKA are implicated as having facilitatory roles aiding glutamate transmission in dorsal horn upon release in response to stimuli of a specific intensity of stimuli. This proposed role for SP and NKA agrees with the evidence presented in section 1.6.2 for a role for these tachykinins in 'wind-up'. The similarity between the two different mutants suggests that the central actions of the tachykinins are mediated primarily via the NK_1 receptor subtype. It is interesting to note that peripheral inflammatory events attributed in part to the action of SP and NKA, such as oedema formation, still occur in both mutant mice. Many different mediators play modulatory roles in inflammation (see figure 1.2), some of these mediators are reported to have similar actions to SP or NKA, for instance bradykinin, histamine and 5-HT are known increase vasodilation and permeability of the microvasculature (Rang and Dale 1991). It is likely that other mediators take over the role of SP and NKA in the absence of these neuropeptides or the lack of NK_1 receptors. Interestingly both PPT-A knockout and NK_1 receptor knockout mutant mice have similar patterns and densities of immunohistochemical staining for CGRP in the spinal cord wild type mice (Cao et al 1998, De Felipe et al 1998), a neuropeptide which has been demonstrated to mediate an number of inflammatory events (see section 1.6).

SP and NKA are widely expressed throughout both the peripheral and central nervous system, as well as in non-neuronal cells of neural crest origin, such as pituitary (Brown et al 1991), adrenal glands (Kuramoto et al 1985), thymus (Ericsson et al 1990), leydig cells (Chiwakata et al 1991) and eosinophils (Weinstock et al 1988). In mutant mice with a disrupted PPT-A gene, the loss of SP and NKA occurs throughout the body and is not localised to primary sensory nerves. Additionally the evidence that tachykinins have a role in proliferation of endothelial cells (Ziche 1990 b, Rosengurt 1991) suggest that they may play a role in development. As a result it can not be certain that the differences between mutant and wild type mice are solely due the loss of SP in sensory nerves, but may reflect a deficiency in a system contributing to the transmission of pain transmission and the development of inflammation.

1.14 NFκB Family of Transcription Factors

NFκB was first described as a nuclear factor capable of binding to the enhancer of the gene encoding the κ light chain of antibodies in B cells (Sen and Baltimore 1986). Since its initial discovery it has been found in a large number of cell types including neuronal and glial cells (Meberg et al 1995, O'Neill and Kaltschmidt 1997). Cloning studies have also shown the existence of at least 5 members of the NFκB family in mammals, namely p50, p52, p65 (also known as RelA), RelB and c-Rel. (Baeuerle and Baltimore 1996). Members of the NFκB family exert their effects on gene transcription by binding to genes in different combinations of hetero- and homodimers. Comparison of NFκB binding sites in inducible genes has led to the proposal of the consensus sequence of κB binding, of $GGGA^{A}/_{C}TN^{T}/_{C}CC$ (Faisst and Meyer 1992). In an inactive state NFκB dimers are found in the cytoplasm complexed

to an inhibitory protein termed IκB, of which there are 5 different forms in mammals termed α, β, γ, δ and ε. The most common cytoplasmic complex is a heterodimer of p50/p65 bound to IκB-α. Phosphorylation of IκB-α at two serine residues (at positions 32 and 36) leads to ubiquitination and subsequent proteolytic degradation of IκB-α, unmasking the nuclear localisation signal of NFκB (Zabel et al 1993, Beg et al 1992). This allows the dimer to translocate to the nucleus and regulate gene transcription. This pathway is described in figure 1.6.

A number of protein kinases have been reported to cause the phosphorylation of IκB *in vitro*, including protein kinase C, PKA and casein kinase (Ghosh and Baltimore 1990), however doubt has been cast on the putative *in vivo* roles of these kinases. However a cytokine-responsive IκB kinase has been purified from HeLa cells treated with tumour necrosis factor α (TNF-α), which shows substrate specificity and kinetics of activation which correlated well with IκB-α phosphorylation in living cells (Di Donato et al 1997).

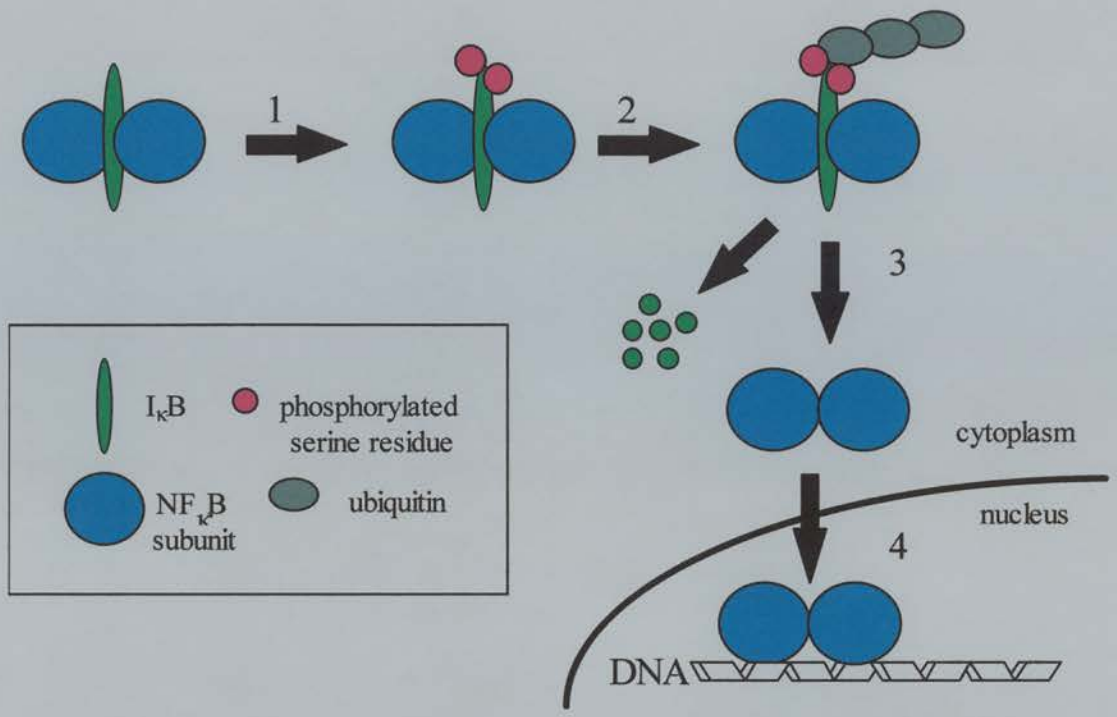


Figure 1.6 Activation of NFκB by phosphorylation induced degradation of IκB

1) NFκB dimer is sequested in the cytoplasm bound to IκB. Phosphorylation of IκB occurs at specific serine residues in response to extracellular stimuli.

2) Phosphorylated IκB is ubiquinated by ubiquitin conjugating enzymes.

3) Ubiquitin labelling marks IκB for degradation by proteasome. Degradation of IκB unmasks the nuclear localisation sequence of NFκB, allowing translocation to the nucleus

4) Once in the nucleus NFκB can influence the transcription of genes with recognition sequences in their promoter regions.

Adapted from Baeuerle and Baltimore 1996

1.15 The STAT Family of Transcription Factors

Studies have shown the transcription of a number of genes is increased within minutes of application of interferon α and γ to cultured cells (Friedman et al 1984, Larner et al 1984). This transcriptional activation is mediated via a novel signalling pathway, central to which are janus kinases (Jak) and signal transducers and activators of transcription (STAT). This pathway is described in figure 1.7.

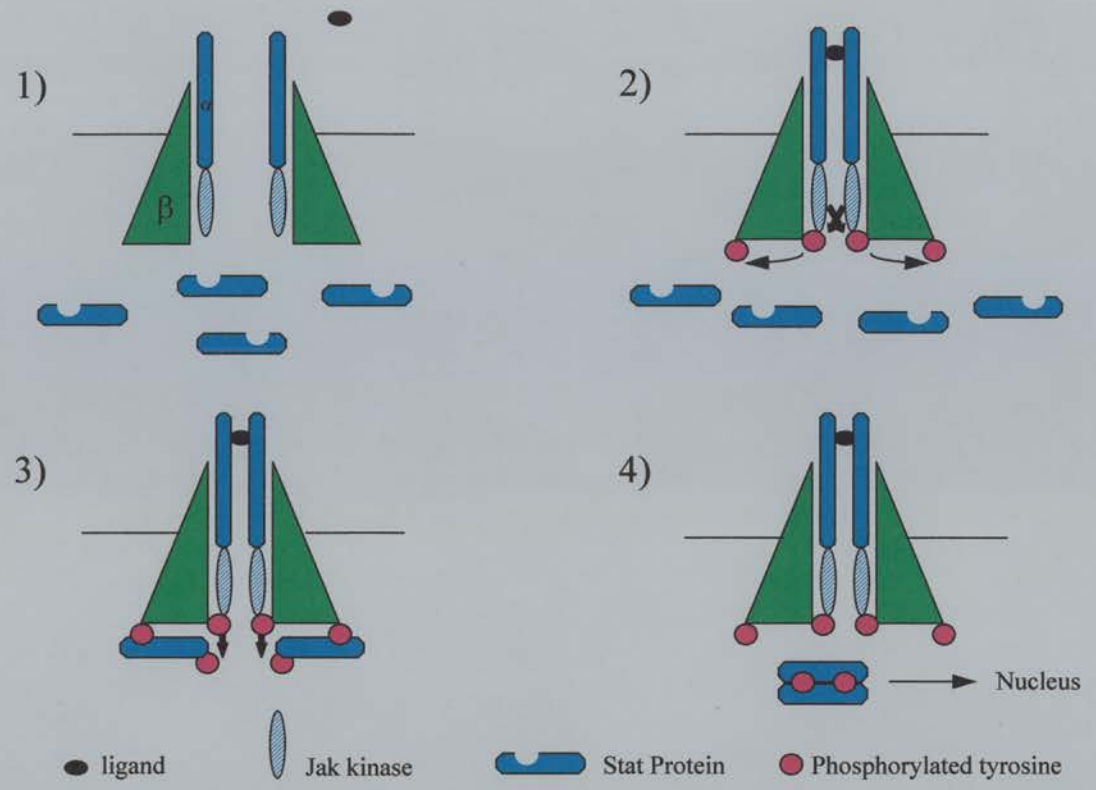


Figure 1.7 The Jak/STAT pathway

1) Jak/Stat associated receptors consist of 1α and 1β subunit, ligand binding induces receptor dimerisation

2) Dimerisation of the receptor brings associated Jak kinases into apposition, enabling a) reciprocal transphosphorylation and b) phosphorylation of a distal tyrosine on the β subunit of the receptor

3) The phosphorylated receptor residue is recognised by the SH-2 domain of a STAT protein, drawing them into the receptor complex, where they are activated by tyrosine phosphorylation

4) The activated STAT proteins form hetero- or homo- dimers which translocate to the nucleus, binding to specific sequences of DNA and modulating transcription.

In this scheme the Jaks can be identical or different, as can the STATs. Modified from Schindler and Darnell (1995).

In mammals there are 4 identified members of the Jak family, Jak1, 2 and 3, and Tyk2, (Ihle and Kerr 1995) which are associated with the cytoplasmic domain of the α subunit of the receptor. Binding of ligand at the associated receptor causes phosphorylation of Jaks at specific tyrosine residues and results in activation of kinase activity (Schindler and Darnell 1995). One of the targets for phosphorylation by activated Jaks are the STAT family of transcription factors. At present 7 STAT proteins have been identified in mammals; namely STAT 1, 2, 3, 4, 5a, 5b, 6, which show a large degree of intra-family sequence homology (Schindler and Darnell 1995). The highest area of conservation is in a region near the carboxyl terminus, called the SH2 domain, which is virtually identical to the SH2 domain of the src kinase family (Ihle and Kerr 1995).

Adding further complexity to the STAT family, two forms of STAT 1 have been identified, encoded by a single gene, an 84 kDa protein (STAT 1α) and a 91 kDa protein, (STAT 1β) (Schindler et al 1992).

Phosphorylation at specific tyrosine residues allows STAT dimerisation and nuclear translocation (Figure 1.6), Mutation of the tyrosine residue at position 701 of STAT 1 prevents its translocation to the nucleus (Gutch 1992). Activated STATs translocate to the nucleus as either hetero- or homodimers capable of binding DNA and activating transcription (Shuai et al 1993, Zhong et al 1994). For example the transcription factor ISGF-3 has been identified as containing STAT 2, either STAT 1α or 1β, and a 48 kDa protein and (Fu et al 1990). Comparison of the promoter region of genes regulated by interferon α or γ revealed that binding occurs at specific sequences of DNA. Genes stimulated by addition of interferon γ contain interferon γ activation sites (GAS), whilst genes stimulated by the addition of interferon-α contain interferon-α stimulated response elements (ISRE). The consensus of these sequences are

characterised in table 1.3.

Interferon γ activation sites (GAS)	TTNCNNNAA
Interferon-α stimulated response elements (ISRE)	AGTTTCNNTTTCN$^{C}/_{T}$

Table 1.3 Consensus sequences of DNA binding sites for STAT proteins, based on Darnell et al 1994.

Further research has demonstrated that the Jak/STAT pathway can be activated by a wide variety of cytokines, growth factors and hormones (Schindler and Darnell 1995). Given the complexity of other signalling pathways it is conceivable that some degree of cross-talk with other signalling molecules could occur, indeed the glucocorticoid receptor (GR) has shown to act synergistically with STAT 5 at the level of transcription (Stocklin et al 1996).

1.16 Aims

Previous studies from the laboratory have used a model of unilateral tibio-tarsal joint inflammation induced by sub-dermal injection of 0.15ml FCA. This stimulus causes an increase in mRNAs from βPPT-A and the α-CGRP encoding gene in ipsilateral small diameter L5 DRG neurones as early as 8h after FCA, no changes occur in contralateral small diameter DRG neurones. The ipsilateral increases are prevented by administration of local anaesthetic around the innervating sciatic nerve prior to FCA injection, suggesting that neural activity may underlie the increases in neuropeptide encoding mRNAs. Initial investigations into putative molecular mechanisms involved in the ipsilateral increases in the expression of PPT-A and the α-CGRP encoding gene have found that mRNA encoding AP-2 increases in L5 DRG 1h after FCA injection. Using the FCA induced model of unilateral tibio-tarsal joint inflammation. The aims of work presented in this thesis are as follows:

1) To study the time course of the levels of mRNAs encoding PPT-A and α-CGRP over the first 8h and to ascertain the possible roles of AP-2 and NGF in the increases in mRNAs seen by 8h after FCA injection.

2) To investigate whether the increases in mRNA is reflected in an increases in hnRNA and hence *de novo gene* expression.

3) To explore the time course of levels of SP and CGRP in L5 DRG, to see if they reflect increases in their encoding mRNAs.

4) To investigate changes in neural activity in sensory nerves after FCA injection, and the requirement for neural activity in the increases in βPPT-A and α-CGRP mRNAs.

5) To examine the development of inflammation over the first 8h after FCA injection, measured by oedema formation, mechanical hyperalgesia and histological changes in the area of injection.

6) To identify putative molecular mechanisms underlying the increased expression of the βPPT-A and α-CGRP encoding genes

2 MATERIALS AND METHODS

All chemicals and solvents used were of analytical grade and were obtained from BDH Chemicals Ltd, Poole, UK or Sigma Chemicals Ltd, Poole, UK, unless otherwise stated. Enzymes and their respective buffers were obtained from Promega Ltd, Southampton, UK unless otherwise stated.

2.1 Induction of Joint Inflammation

Unilateral joint inflammation was induced in male Han-Wistar rats (200-300g) by intra-dermal injection at two sites around the left tibio-tarsal joint with 0.15ml of 1mg/ml Freund's Complete Adjuvant (FCA) containing 150μg *M. tuberculosis*, whilst under 4% halothane anaesthesia. Control animals received no injection or 0.15ml sterile saline injected as described for FCA.

2.2 Removal of L5 Dorsal Root Ganglia

Animals were killed by CO_2 inhalation, at specific time points and L5 DRG innervating the limb ipsilateral and contralateral to injection were identified by tracing the sciatic nerve, which branches into DRG L4-6. DRG were removed by laminectomy, snap frozen on dry ice and stored at -80^0C

2.3 In-situ Hybridisation

2.3.1 Solutions for in-situ hybridisation

Diethyl pyrocarbonate (DEP) water	0.02% DEP in distilled water, leave to stand for 2 hours and sterilise by autoclaving
4% Paraformaldehyde	20mM NaH_2PO_4, 80mM Na_2HPO_4, 40g paraformaldehyde in 1 litre DEP water
20xSSC (sodium saline citrate)	3M NaCl, 0.3M trisodium citrate
RNase free 2xSSC (for postfixation rinsing)	50ml RNase free 20xSSC diluted with 450 ml DEP water
2x Hybridisation buffer	1.2M NaCl, 20mM Tris-Cl (pH 7.5), 2mM EDTA, 20% (w/v) dextran sulphate, 2xDenhatrs, 0.2mg/ml salmon sperm DNA, 0.25mg/ml yeast tRNA (Gibco BRL Life Technologies Ltd, Paisley, UK)
Deionised formamide	Mix 15 g Amberlite ion exchange resin with 150 ml formamide for 1 hour at room temperature and filter.
Box Buffer (for hybridisation)	50% deionised formamide, 20% 20xSSC, 30% DEP water
RNase Buffer	0.5M NaCl, 0.01M Tris-Cl, 0.01mM EDTA
TBE (10x)	108.9g Tris base, 55.7g boric acid, 4.7g EDTA made up to 1 litre
4% Polyacrylamide gel for electrophoresis	3.6g Urea, 1.32ml 40% acrylamide:bis-acrylamide (19:1), 1ml 10xTBE, distilled water to 10ml. Polymerise with 100µl 10%w/v ammonium persulphate and 10µl TEMED (N,N,N',n'-tetramethylethylenediamine)

TE Buffer (1x)	10mM Tris-HCl (pH 8.0), 1mM EDTA
1% agarose gel	1% w/v agarose in 0.5xTBE, dissolved by heating in microwave oven and 1μg/ml of ethidium bromide added

2.3.2 Tissue sectioning

Glass microscope slides were prepared for tissue mounting by 3 minute washes in each of 0.2M HCl/DEP water, DEP water, and acetone and drying in an oven at 60^0C. Slides were then coated in a subbing solution of 0.15% gelatin, 0.03% sodium azide in DEP water for 5 minutes, dried and then coated in 0.2% poly-L-lysine in DEP water. DRG sections were cut to 10μm thickness using a -20°C Cryostat (Bright Instrument Company Ltd, Huntingdon, UK), and mounted on subbed glass microscope slides. Cut sections were stored at -80^0C.

2.3.3 *In-vitro* transcription of complementary riboprobes

Plasmid constructs containing cDNA template sequences encoding βPPT-A (454bp full length cDNA a kind gift from Prof A.J. Harmar, cloned into pGEM3 (Promega)) and α-CGRP (450bp fragment encoding 3' untranslated sequence a kind gift from Dr S. Amara, cloned into pSP64) were linearised by digestion with the restriction enzyme EcoR1 to allow *in vitro* transcription as follows; 10μg plasmid in TE (pH8), 2 units EcoR1, 1x EcoR1 reaction buffer (90mM Tris-HCl, 10 mM $MgCl_2$, 50mM NaCl), 28μl DEP water. Digestion was carried out at 37^0C for 1h, and complete digestion and hence linearisation verified by electrophoresis on a 1% agarose gel in 0.5xTBE buffer. DNA was visualised on a UV transilluminator.

Linearised templates were incubated in a total volume of 10μl, containing 0.5mM

ATP, CTP, GTP and a ratio of ^{35}S-UTP (800 Ci/mmol, Amersham International plc, Amersham, UK) and cold UTP to a specific activity of 3-5x10^8. 10mM DTT, RNase inhibitor (Gibco BRL), 1x transcription buffer (40mM Tris-HCl, 6mM $MgCl_2$, 2mM spermidine, 10mm NaCl) and 1U SP6 polymerase at 40^{0}C for 1h. Transcription was terminated by application of 1 unit DNase1 (RNase free, Gibco BRL) for 10 min at 37^{0}C. Generated probes were then extracted either by;

1) eluting down a NICK column (Pharmacia Biosystems Ltd, Milton Keynes UK)

2) isolation with phenol:chloroform:isoamyl alcohol, and subsequent precipitation of the RNA with 1µl glycogen, 0.5M ammonium acetate and 2.5x vol ethanol (Hayman Ltd, UK) at -80^{0}C for 10 min. The pellet was recovered by centrifugation at 14,000 rpm, dried under vacuum and resuspended in DEP water.

The amount of β-emitting radioactivity in 1µl of the manufactured probe was counted by scintillation, to give an estimate of the total activity of the probe. To check for incorporation of nucleotides and degradation of the probe 1µl was electophoresed on a 4% polyacrylamide denaturing gel run in 1xTBE buffer and exposed to β-max film. Successfully made probes were stored at -20^{0}C.

2.3.4 Hybridisation and washing

Cut sections were removed from -80^{0}C storage and postfixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 10 min. This was followed by 3x5 min washes in RNase free 2xSSC containing diethylpyrocarbonate (0.02%). The synthesised cRNA probes were denatured by heating to 70^{0}C and added to hybridisation buffer to give 10x10^6 counts/ml. 200µl of this hybridisation

mix was added to each slide and hybridisation carried out overnight in sealed containers at 50^0C, humidified with box buffer. Slides were subjected to 3x5 min washes in 2xSSC, before incubation with RNase A (30μg/ml in RNase buffer) for 1h at 37^0C. This was followed by a washes in 2xSSC for 10 min, and 0.01xSSC at 55^0C and room temperature for 1h each. Sections were dehydrated with graded ethanol (50%, 70%, 90%) in 0.3M sodium acetate, air dried and dipped in NTB2 autoradiography emulsion (Kodak, UK) diluted 1:1 in distilled water at 42^0C. Sections were exposed for 2-3 weeks at 4^0C, counterstained with haemotoxylin and eosin and coverslipped with DPX mountant. Sections were viewed at 20x magnification by microscopy, allowing mRNA expression to be visualised as individual silver grains. Individual silver grains per section were quantified in the different diameters of sensory neurones, see section 1.2.2, using circles with diameters of 20μm and 40μm and computer aided image analysis (Seescan, Cambridge, UK). Background counts, measured from non-expressing regions of the section using the 20μm and 40μm diameter circles, were averaged and subtracted from the respective foreground counts, to negate the effects of non-specific probe binding. Total cells and number of clearly expressing cells within both sizes of neurones were counted by eye under 20x magnification in order to calculate the proportion of expressing neurones.

2.3.5 Role of protein synthesis in increases in α-CGRP encoding transcripts

To investigate whether the observed increases in α-CGRP encoding mRNAs were dependent on the de novo synthesis of a protein, such as a transcription factor, cycloheximide was injected systemically at a non-lethal dose (3mg/kg in sterile

saline, i.p. Rothblum et al 1976). 60 min after cycloheximide administration animals were injected with FCA (as described in 2.1) and killed 30 min thereafter. Positive controls received FCA and were sacrificed 30 min after injection. The effects of cycloheximide injection alone were studied in animals receiving cycloheximide, but no FCA, these animals were killed 90 min after cycloheximide injection. α-CGRP encoding mRNAs were quantified in DRG neurones with diameters ≤ 20μm as described above.

2.3.6 Statistics

Experimental in-situ hybridisation data is expressed as percentage of untreated control values and presented as mean±S.E.M. and compared by ANOVA followed by Dunnett's post hoc test. The Null hypothesis was rejected at the 5% level.

2.4 Sub-cloning of Intronic Sequence of βPPT-A

mRNA levels in a tissue are dependent on the rate of production and rate of breakdown, thus quantification of mRNA as described above only gives steady-state levels. The production of new mRNA due to induction of genes can be assessed by measuring levels of hnRNA. This can be achieved by in-situ hybridisation using a probe to an intronic sequence of the gene of interest, the subcloning of a probe for an intronic sequence of βPPT-A is described below.

2.4.1 Solutions

LB (Luria-Bertoni) broth	1% w/v bacto-tryptone, 0.5% w/v yeast extract (both Difco Laborities, Michigan,USA), 0.5% w/v NaCl and

	autoclave
LB agar	As LB broth except with 1.5% (w/v) bacto agar (Difco Laborities) and autoclave
LB agar plates	LB agar was melted in the microwave, allowed to cool until warm, and if required ampicllin (100mg/ml was added prior to pouring into sterile plastic 100mm petri dishes
GTE buffer	50mM glucose, 25 mM Tris-HCl (pH 8.0), 10mM EDTA
Potassium acetate	Prepared by combining 60 ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml water. The final solution is 3M with respect to potassium, 5M with respect to acetate
1% low melting point agarose gel	As for 1% agarose gel, but using low melting point agarose (Gibco BRL)

2.4.2 Generation of insert

An approximately 700bp fragment within intron E of βPPT-A was produced by BamH1 and HindIII digestion of a genomic clone of rat βPPT-A (Carter and Krause 1990) in 1x restriction buffer (6mM Tris-HCl, 6mM $MgCl_2$, 100mM NaCl, 1mM DTT) at 37^0C for 1h. Resulting DNA fragments were separated by size using electrophoresis on a 1% low melting point agarose gel. The band corresponding to 700bp was cut from the gel and extracted from the gel using a Hybaid recovery DNA purification kit (Hybaid Ltd, Teddington, UK).

2.4.3 Ligation of insert into pGEM3

pGEM3 was cut with BamH1/HindIII and purified as described 2.4.1. The 700bp fragment was ligated into pGEM3 using T4 DNA ligase and 1mM ATP in 1x ligase buffer (30mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT) overnight at 37^0C, at ratios of fragment to vector at 8:1 and 12:1 to optimise conditions.

2.4.4 Preparation of competent bacteria

A single colony of HB101 *Escherichia coli* was inoculated into 2ml of LB broth and grown overnight in a shaking incubator at 37^0C. Cells were subsequently resuspended in 40ml LB broth and grown to mid-log phase (A_{600}= 0.4-0.8) the resulting cells were then harvested by centrifugation in a Beckman JA20 rotor/J2MC centrifuge at 6000rpm, 4^0C for 5 min. Cells were resuspended in 10ml ice cold 0.1M $CaCl_2$ and placed on ice for 10min to 1h. The cells were collected by repeating centrifugation and resuspended in 2ml 0.1M ice cold $CaCl_2$. Cells were stored on ice until used, a maximum of 4 days later.

2.4.5 Transformations

The method for this stage follows the protocol set out by Sambrook et al, 1989. 100µl of competent cells were mixed with 10-100ng of plasmid DNA and placed on ice for 10 min, prior to heat shock at 42^0C for 90 seconds. Transformed cells were plated out onto LB agar plates containing 100µg/ml ampicillin with an ethanol flamed glass spreader. Plates were inverted and incubated overnight at 37^0C. Plates for positive control, pGEM3, and negative control, with no plasmid, were run in parallel. Viable colonies were those which had been transformed into plasmids containing an ampicillin resistance gene, β-lactamase.

2.4.6 Small scale preparation of plasmid DNA

To check for successful insertion, a colony was taken from the plate, suspended in 2ml LB broth containing 100μg ampicillin and grown overnight at 37^0C in a rotator. Bacterial pellets were centrifuged in eppendorf tubes for 30 s and resuspended in 100μl GTE buffer. Cells were lysed with 200μl 1%SDS/0.2M NaOH and neutralised with 150μl potassium acetate. DNA was isolated by precipitation with phenol:chloroform, and resuspended in TE/RNase. 5μl was cut with Hind III/ Bam H1 as described above and run on 1% agarose gel to verify successful insertion. Cells from a colony with the inserted fragment were transferred to a plate and grown overnight at 37^0C. The remaining cells were added to 500 ml LB/500μl ampicillin (100mg/ml), and grown overnight at 37^0C in a rotator.

2.4.7 Large scale preparation of plasmid DNA

Cells were harvested at 6,000 rpm for 5 min at 4^0C using a Beckman J-14 rotor in a Beckman J2-MC centrifuge. The resulting bacterial pellet was resuspended in 12ml GTE, mixed well with 24 ml freshly prepared 1%SDS/0.2M NaOH and stored on ice for 5 min. 16 ml of ice cold potassium acetate was added, mixed, and left on ice for a further 10 min, prior to centrifugation at 6,000 rpm for 10 minutes at 4^0C in the J-14 rotor. The resultant supernatant was strained through gauze into a fresh 250ml centrifuge pot, 32 ml isopropanol added and left at room temperature for 30 min whilst the DNA was precipitated. The plasmid DNA was pelleted by centrifugation at 10,000 rpm for 3min at 4^0C. The pellet was air dried and resuspended in 2.2ml TE. 2.7g CsCl was added, and dissolved, and then 100μl ethidium bromide added. This mixture was transferred to a 3ml Beckman Quickseal tube and the weight made up to

5.8-6g by the addition of a saturated solution of CsCl in TE. Centrifugation was carried out using a TLA100.3 rotor in a Beckman Optima TLX Ultracentrifuge either at 100,000 rpm for 4h or at 70,000 rpm overnight. Banded plasmid DNA was removed using a needle and syringe through the tube wall, and transferred to a fresh ultracentrifuge tube and CsCl in TE was added and recentrifuged as described above. The resultant band was extracted as before and the ethidium bromide removed using isopropanol, until the isopropanol was no longer pink. CsCl was removed from the extracted plasmid preparation was dialysed against two 1 litre changes of TE at 4^0C. Concentration of the recovered plasmid DNA was evaluated by measuring absorbency at A_{260} in a Shimadzu UV-160A spectrophotometer, the absorbance of 50μg/ml DNA being 1.0 at A_{260}. Plasmid DNA was stored at -20^0C.

In situ hybridisation using ^{35}S-labelled cRNA probes generated from the plasmid containing sequence from intron E of βPPT-A, termed pDB1, was carried out in DRG neurones with diameter≤ 20μm as described in 2.3. 30 min after FCA injection. Data was analysed by ANOVA followed by Dunnett's post hoc test, the Null hypothesis was rejected at the 5% level.

2.5 Protein Levels of SP and CGRP after FCA Administration

2.5.1 Peptide extraction

Joint inflammation was induced as previously described and innervating L5 DRG dissected from rats 30 min, 1,2,4 and 8h after adjuvant injection and stored at -70^0C. Peptides were extracted from L5 DRG by acid hydrolysis with 2M acetic acid, and samples evaporated to dryness by centrifugation under vacuum in a Savant DNA 110

Speed Vac, and resuspended in 500µl radioimmunoassay buffer (0.1M sodium phosphate, 0.05M sodium chloride, 0.1% bovine serum albumin, 0.01% sodium azide, pH 7.4) and stored at -70^{0}C.

2.5.2 Radioimmunoassy

RIA was carried out using kits for SP and CGRP (Peninsula Laboraties, USA) as follows: 100µl of peptide standards (0.1-128pg) or sample were incubated with 100µl rabbit primary antibody at 4^{0}C for 16-24h. 15,000 cpm of ^{125}I-peptide in 100µl was added and incubated at 4^{0}C for a further 16-24h. Bound and free radiolabel was separated by precipitation with 100µl goat anti-rabbit immunoglobulin and rabbit serum. Supernatants were removed by aspiration and samples counted in a beta-counter. The following internal controls were carried out; total counts, 200µl RIA buffer and 15,000 cpm of ^{125}I-peptide but was not aspirated; non-specific binding, 200µl RIA buffer and 15,000 cpm of ^{125}I-peptide; total binding, 100µl RIA buffer, 100µl rabbit primary antibody, and 15,000 cpm of ^{125}I-peptide.

Experimental data is expressed as percentage of untreated control values and presented as mean±S.E.M. Data was analysed by ANOVA followed by Dunnett's post hoc test. The Null hypothesis was rejected at the 5% level.

2.6 The Role of Sensory Neural Activity in FCA Induced Joint Inflammation

2.6.1 Neural recordings from small diameter sensory nerves

The early effects of 0.1 ml FCA intra-articular injection into the knee joint, which shares a common innervation with the tibio-tarsal joint, were studied. Rats were

anaesthetised with urethane (ethyl carbamate 25% w/v, 6 ml/kg body weight i.p.). Neural discharge from afferent fibres innervating the knee joint was measured by exposing the medial articular nerve where it branched from the saphenous nerve and dissecting it from muscle and connective tissue, rupturing cutaneous branches of the medial articular nerve, thereby restricting neural recordings to fibres innervating the joint capsule and surrounding tissue. The exposed nerve was covered in liquid paraffin, cut centrally to abolish efferent nerve activity and recordings were taken from fine nerve filaments containing 1-3 active units using bipolar platinum-iridium (90:10) electrodes. The electrical signal was amplified (Neurolog NL103 and 105 amplifiers) and displayed on a storage oscilloscope (Tektronix 5113). The signal was digitised using a digital audio processor (Sony PCM 701-ES) for storage on videotape (videotape recorder: Sony Betamax SL-HF100UB). The output from the amplifier was passed through a filter (NL115 10Hz-1KHz) and a voltage discriminator (Digitimer D130). Action potentials from that fell within the selected window, defined as the response of a polymodal C fibre to capsaicin (3μg i.a.) and/or mechanical probing of the joint capsule, generated an output pulse that was counted by a PC using software developed in-house. Experiments were conducted by D. Kelly.

2.6.2 Requirement of neural activity for the increases in βPPT-A and α-CGRP encoding mRNAs

To investigate whether the increase in neural activity following FCA injection underlied the increases in βPPT-A and α-CGRP encoding mRNA, levels of these transcripts were measured in L5 DRG ipsilateral to FCA injection in rats pre-administered with local anaesthetic. 2% lignocaine was injected around the sciatic

nerve, in the mid-thigh region. Animals which showed a paralysis of the limb 30 minutes after injection were subsequently injected with FCA as described in section 2.1 and in-situ hybridisation carried out as described in section 2.3.

2.7 Progression of Inflammatory Indices

Oedema formation was monitored by measurement of tibio-tarsal joint circumference immediately before (t=0) and 30 min, 1, 2 4 and 8h after injection of FCA (as described in section 2.1). The development of mechanical hyperalgesia was assessed at the same time points, by pressure applied to the tibio-tarsal joint required to induce reflex withdrawal of the limb. Measurements were made using a Grass Force-Displacement Transducer connected via a Bridge amplifier and Mac Lab 4S (both ADI instruments) to a Power Mac G3 running Chart software. 3 measures per time point were taken and averaged. Data was analysed by ANOVA followed by Dunnetts Post hoc test ($p>0.05$), the Null hypothesis was rejected at the 5% level.

2.8 Histology of Tibio-tarsal Joints at Time Points After FCA Induced Joint Inflammation

Untreated animals and animals 1, 8 and 24h after FCA injection were anaesthetised with sodium pentobarbitone (60mg/kg i.p.) and perfused via the left ventricle with heparinised saline (500 U/kg, 10ml), followed by 10% formol saline (50-100 ml), fixing the hind limbs. Hind limbs were removed and stored in 10% formol saline. Tibio-tarsal joints were fixed in 40% buffered formalin and processed into paraffin wax. Sections were cut at 3-5μm, placed onto glass slides and stained with

haematoxylin and eosin.

2.9 Antisense Oligonucleotides

Oligonucleotides were designed following sequence analysis of β-PPT A and α-CGRP and application of the guidelines for design set out in section 5.1. Sequences were cross-referenced with pre-existing sequences using computer software available at the UK MRC Human Genome Mapping Project Resource Centre (HGMP). Oligonucleotides were synthesised with a backbone of phosphorothioate bases, each oligonucleotide was 17 bases long (Oswel, Southampton, UK). The oligonucleotides used are shown in table 2.1:

PPT ATG	5' ATT TTC ATG TTG GAT TT 3'
PPT Exon 2	5' CAA TAA TTT AGA TCA TC 3'
CGRP ATG	5' TTC AGA AAG CCC ATG AT 3'
Scramble	5' GTT ATC TTT GTA TGT AT 3'

Table 2.1 Sequences of antisense oligonucleotides applied to cultures of DRG neurones

Cultures of adult rat dorsal root ganglia

Rats, two per experiment, were killed by chloroform, and the spinal cord removed under sterile ethanol conditions. The spinal cord was split exposing the DRG (40-45), which were removed under microscope and placed in HAMS F-14 serum free media, containing 4% Ultroser G (Gibco BRL), 1% penicillin/streptomycin (Gibco BRL), 0.5% L-glutamine. The ventral root was separated from ganglionic body, which was transferred to a tissue culture dish, the medium removed and replaced by 1.8ml HAMS F-14 and 200μl 10X collagenase. After 3 hours the DRG were

removed and washed with serum free HAMS F-14 medium, spun in a centrifuge and resuspened in 1.8ml serum free HAMS F14 and 200µl trypsin at 37^{0}C for 30 minutes. Trypsin action was stopped by addition of HAMS F14 medium containing serum, cells resuspended in 3ml of HAMS F14 and pulped by trituration into a suspension. Cells were left overnight to attach to a plate previously coated with polyornithine (500µg/ml). The media, containing non adherent cells and dead cells, myelin and axonal debris and non neuronal matter, was removed. Neurons only lightly attach to plates, so can be removed by gentle pipetting of serum containing media, leaving firmly attached non-neuronal cells on the plate. Neuronal cells were plated to 10,000 cells per well coated with polyornithine (500µg/ml) and laminin (5µg). 75µl of HAMS F-14 medium as described above and mouse salivary gland derived NGF (50ng/ml) was added to each well. Neuronal cell cultures were maintained at 36.5^{0}C, with 97% air:3% CO_2 and in the presence of cytosine arabinose (10µM), to prevent cell division and the proliferation of non-neuronal cells, and grown overnight.

Each antisense oligonucleotide (AS-ON), at concentrations of 2.5µM and 500nm in serum free medium, was applied to DRG cultures and incubated for 18h at 37^{0}C. The efficacy of each AS-ON treatment was assessed by levels of SP and CGRP extracted from each culture by hydrolysis with 2M acetic acid, and stored at -20^{0}C until quantified by radioimmunoassay.

Radioimmunoassay

Samples were evaporated to dryness in a vacuum oven (45^{0}C, 700mmHg) and resuspended in assay buffer (0.1M PO_4, pH 7.6, 1g/l bovine serum albumin). For SP

assay, standards (0-500 pg/ml) and samples were prepared in a volume of 200µl in duplicate, and incubated at 4^0C for 16 h with 200µl rabbit anti-SP antiserum (1:80,000 dilution) and 5000cpm in 100µl of ^{125}I labelled SP. CGRP radioimmunassay was performed with standards ranging from 0-5000 pg/ml of human CGRP (Peninsula Laborities, USA). Standards and samples were incubated for 16h at 4^0C with 100µl of 1:2000 dilution of rabbit anti-human CGRP antiserum (Amersham Life Sciences) and 5000cpm 2-[^{125}I] iodohistidyl CGRP (human, Amersham Life Sciences). Bound radiolabel was separated from free label by precipitation with donkey anti-rabbit gamma globulin antiserum (Scottish Antibody Production Unit (SAPU), Carluke, UK).

Experimental data was analysed by ANOVA followed by Dunnett's post hoc test, the Null hypothesis was rejected at the 5% level.

2.10 Western Blots

Solutions

1x Tris-Glycine Electrophoresis Buffer	2.5mM Tris-HCl, 19.2mM glycine, 0.1% w/v SDS
1x Tris-Glycine Electroblotting Buffer	2.5mM Tris-HCl, 19.2mM glycine, 2% methanol
10% Resolving Gel	5 ml 40% acrylamide, 3.0ml 1.875M TrisHCl (pH8.8), 150µl 10% sodium dodecyl sulphate. Make up to 15ml with distilled water. Polymerise acrylamide with 50µl 10% ammonium persulphate (Sigma) and 7.5µl TEMED
Stacking Gel	0.8ml 40% acrylamide, 0.5ml 1.25M TrisHCl (pH6.8), 50µl, 10% sodium

	dodecyl sulphate. Make up to 5ml with distilled water. Polymerise with 17µl 10% ammonium persulphate and 5µl TEMED
Gel Loading Buffer	5.8ml glycerol, 2.5ml 1.25M TrisHCl (pH6.8), 2.5ml 2-mercaptoethanol, 1g SDS, 5mg bromophenol blue, and 35ml distilled water

Sample Preparation

L5 DRG were removed from untreated animals as controls. Single DRG were homogenised in 60µl loading buffer, boiled for 5 minutes and spun at 13,000 rpm for 10 min. Samples (20µl) were run alongside recombinant p65 and IκB-α proteins and pre-stained molecular weight markers between 200,000 and 7,100 kDa on a 10% resolving acrlyamide gel, over laid by stacking gel solution Gels were run at 100v, 20 mA in 1x Tris-glycine electrophoresis buffer. Proteins were transferred by electrophoresis to an Hybond ECL nitro-cellulose membrane (Amersham Life Sciences) activated by immersion in methanol, in 1x Tris-glycine electrophoresis buffer. The membrane was blocked for 1 hour with a 3% powdered milk (Marvel, Premier Beverages, Stafford, UK), 0.1% Tween (polyoxyethylenesorbitan monolaurate) in phosphate buffered saline (PBS) and then probed for 1h with a monoclonal mouse antibody raised against IκB-α, diluted 1:1000 in. Unbound primary antibody was removed with 1x15min and 2x5 min washes with 0.1xTween/PBS. Specific binding was visualised by probing for 1h with a secondary horseradish peroxidase tagged anti-mouse antibody (Scottish Antibody Production Unit, Carluke, UK), diluted 1.5:10000 in 0.1%Tween/PBS, and application of

reagents from an ECL detection kit (Amersham Life Sciences). The recombinant proteins and monoclonal IκB-α antibody were kind gifts from Prof. D Hay (University of St Andrews, UK)

2.11 Identification of STAT Transcripts in DRG by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Extraction of RNA

RNA was extracted from DRG using the guanidium thiocynate method as previously described by (Chomczynski and Sacchi 1987). One DRG was homogenised in 500µl denaturing solution (4M Guanidium thiocyanate, 0.025M sodium citrate, 0.5% sarcosyl, 0.1M β-mercaptoethanol), 50µl 2M sodium acetate added and vortexed. After the addition of 500µl water saturated phenol, and 100µl chloroform:isoamyl (24:1), samples were cooled on ice for 15 min and centrifuged for 20 min at 14,000 rpm. The upper aqueous phase was removed and the RNA in solution precipitated by the addition of 200µl isopropanol and storage at -20^0C for at least 1h. The precipitated RNA was collected as a pellet by centrifugation for 20 min, and the supernatant removed. The pellet was washed with 75% EtOH, dried and resuspended in 50µl DEP water. The concentration of RNA was deduced by absorbance spectrometry at 260 and 280nm in a Genequant RNA\DNA Calculator (Pharmacia Biotech). RNA was also extracted from liver, by the same method, for use as a positive control.

Reverse transcription

Using the Promega reverse transcription system cDNAs were produced by adding 1μg of RNA to the following reaction mixture to a total volume of 10μl: 5mM magnesium chloride, 10mM Tris-HCl (pH 8.8), 50mM KCl, 0.1% triton, 1mM dNTPs, 1 unit/μl RNase inhibitor, 15 unit/μg AMV reverse transcriptase, 0.05μg/μl oligo $(dT)_{15}$ primer. The mixture was incubated at 42^0C for 45 min.

Polymerase chain reaction (PCR)

cDNAs produced by reverse transcription were amplified by polymerase chain reaction in a Hybaid Omnigene PCR machine using 1U Taq DNA polymerase (Boehringer Mannheim UK (Diagnostics/Biochemicals) Ltd Lewes, UK), 400nm degenerate primers designed to amplify the conserved SH-2 domain of STAT 1,3 and 4 (5' AGC TCT AGA $^{T}/_{C}$TN CCT GT$^{C}/_{G}$ GTG $^{G}/_{A}$TG AT$^{C}/_{T}$ TC 3' and 5' TGA CTC GAG CTG AAT CT$^{T}/_{A}$ A$^{A}/_{G}$C A$^{G}/_{A}{}^{G}/_{A}$ AA$^{T}/_{C}$ GTC CC 3' a kind gift from C. Watson) 200μm dNTPs, 1.5mM $MgCl_2$, 50mM KCl, 20mM Tris-HCl (pH 8.4) to a final volume of 50μl in ultrapure water. PCR cycles were performed as follows, 5min at 95^0C; 30 cycles of 30^0C at 95^0C, 1:30 at 60^0C, 2min at 72^0C; 1 cycle of 8 min at 72^0C.

As a positive control PCR using primers for the ubiquitously expressed, structural protein β-actin (5' ATGGATGACGATATCGCTC 3' and 5' ATGAGGTAGTCTGTCAGGT 3') was carried out as detailed above under the following cycles: 5 min at 95^0C; 35 cycles of 45 s at 96^0C, 1:30 min at 60^0C, 2 min at 72^0C;

Cloning of PCR products

A specific band, found in both DRG and liver extracts was excised from a 1% low melting point agarose gel containing ethidium bromide, under UV illumination and DNA extracted using a Hybaid recovery DNA purification kit (Hybaid Ltd). Excised bands were ligated into pGEMT-Easy (Promega Ltd) using T4 DNA ligase and 1mM ATP in 1x ligase buffer (30mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT) at 4^0C overnight and transformed into JM109 High Efficiency Competent Cells (Promega Ltd) by heat shock as follows; the cells were transferred from 20 min on ice to a water bath at 42^0C for 45 seconds and then back to ice for 2 min. SOC medium (2g Bacto-tryptone, 0.5g bacto yeast extract, 1m1 1M NaCl, 0.25ml 1M KCl, 1ml 2M Mg^{2+}, 1ml 2M glucose, to 100ml with distilled water), was added to the cells and incubated for 1.5h at 37^0C in a rotator within an incubator. Cells were then plated on LB plates and grown overnight at 37^0C. DNA was extracted from a single colony as described in section 2.4.5. and cut with EcoR1, for 1h at 37^0C, to check for inserts.

Sequencing of cloned PCR products

DNA extracted from a colony which contained an insert was sequenced using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham). Four reactions were set up, each containing 0.5µl of a different ^{33}P labelled diodeoxyribonucleotide (ddNTP) and 2µl of a mix of the four deoxyribonucleotides (dNTP). To each of these four tubes 4.5µl of a mixture (2µl reaction buffer, 1.5µg DNA, 2pmol 5' primer, 2µl Thermo Sequenase polymerase (4U/µl) water to 20µl) was added, and placed in a Hybaid Omn-E thermal cycler and subjected to the following program, 95^0C for 30 seconds, 55^0C 30s, 72^0C for 90s and repeated for 40

cycles (sequencing performed by Val Lyons). The addition of 4µl of stop solution to each tube terminated the reaction, and samples stored at -20^{0}C. Samples were run on a 6% acrylamide/urea gel, which was made as follows; 5.7g acrylamide, 0.3g bis-acrylamide, 45g urea, 5ml 20x glycerol tolerant gel buffer (216g Tris base, 72g taurine, 4g $Na_2EDTA.H_2O$, H_2O to 1litre) per 100ml. Acylamide was polymerised by 10% ammonium persulphate and TEMED. Gels were run for 4.5h at 1.8kV and exposed to film (X-OMAT AR, Kodak, UK) for 48 h.

3 PLASTIC CHANGES IN SYNTHESIS OF NEUROPEPTIDES IN SENSORY NERVES FOLLOWING PERIPHERAL INFLAMMATION

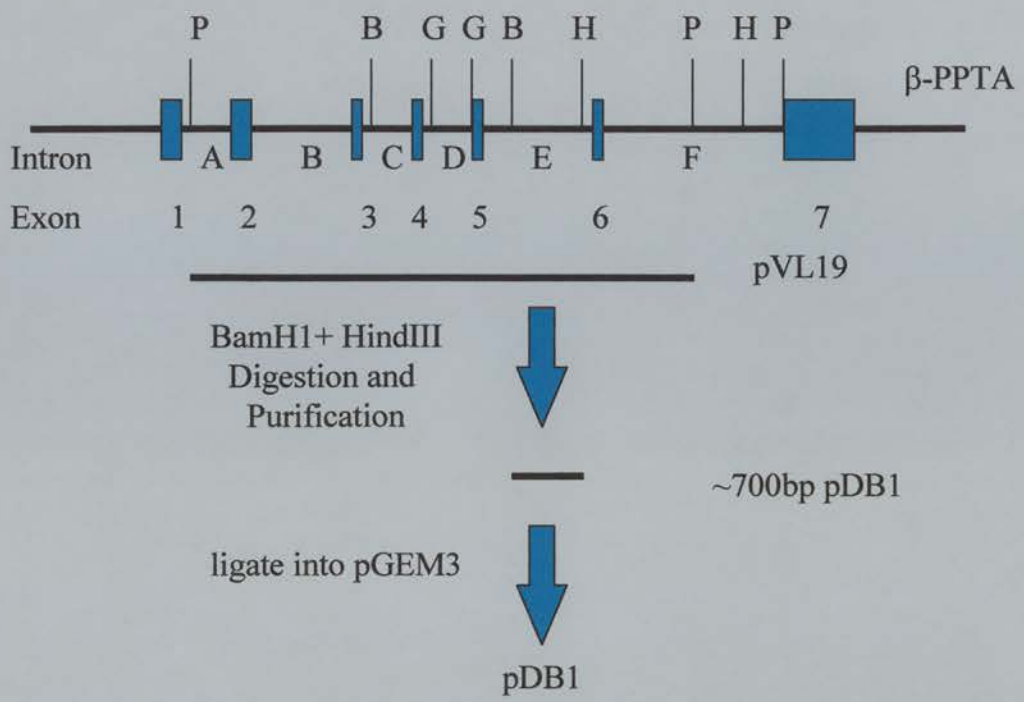
3.1 Introduction

As detailed in chapter 1.6, SP, NKA and CGRP have been implicated in both central and peripheral events following inflammation. The control of sensory neuropeptide biosynthesis in the cell bodies of sensory neurones is of considerable interest and relevance in the aetiology of the inflammatory process. Previous experiments in the laboratory have shown that within 8h of FCA injection SP and CGRP encoding mRNAs are increased in L5 DRG in cell bodies of small diameter nerve fibres innervating the injected joint. In contrast, mRNAs encoding vasoactive-intestinal peptide (VIP) or somatostatin (SOM), are unaffected by FCA injection (Donaldson et al 1992). The molecular mechanisms underlying these changes are unknown, however within 1h of FCA injection mRNA encoding the transcription factor AP-2 was increased, then decreased to control levels at subsequent time points (Donaldson et al 1995 b). mRNAs encoding members of the AP-1 family, NGFIA or NGFIB were not detected or showed no changes in expression within 8h of FCA injection. Intriguingly, EMSA analysis has revealed an active AP-2 site in the promoter region of the rat PPT-A gene between bases -865 and -47 (Quinn et al 1995), suggesting that AP-2 could regulate PPT-A expression.

There is a growing body of evidence that there are also plastic changes within large diameter fibres, responsible for relaying low threshold, innocuous stimuli such as touch and pressure, in models of inflammation. For example the number of Aβ fibres

expressing SP increases 48h after inflammation induced by injection of turpentine oil into the hind paw (Neumann et al 1996).

An exploration of the timecourse of neuropeptide expression following FCA injection may give an indication of the molecular mechanisms underlying plasticity in gene expression. mRNA in-situ hybridisation (ISH) offers a technique for quantifying βPPT-A and α-CGRP mRNAs in the different populations of neurones found in DRG (described in 1.2.2). Furthermore the use of probes complementary to intronic sequences can be employed to quantify hnRNA levels and hence *de novo* gene expression. A probe complementary to a sequence from intron E of βPPT-A was generated (as displayed in figure 3.1) to investigate the *de novo* expression of βPPT-A.



P=Pst1, B=BamH1, G=Bgl11, H=HindIII

Figure 3.1 Construction of pDB1, an approximately 700bp sequence from intron E of βPPT-A, which was subsequently inserted into pGEM3, from which ^{35}S labelled cRNA riboprobes were synthesised and used to quantify *de novo* βPPT-A expression.

3.2 Results

3.2.1 βPPT-A and α-CGRP mRNA expression of in small diameter DRG neurones

Photomicrographs of PPT mRNA expression are displayed in figures 3.2 and 3.3. In-situ hybridisation studies showed that within 30 min of adjuvant injection there are marked rises in expression of both βPPT-A (figure 3.4) and α-CGRP (figure 3.5) encoding mRNAs per small diameter neurone (diameter ≤20μm) in innervating DRG. After 60 min, neuronal expression of both transcripts had approximately doubled compared to either untreated controls or contralateral DRG, which showed no changes throughout the first 8h of inflammation. Significantly elevated βPPT-A and α-CGRP encoding mRNA levels per expressing neurone were maintained up to 8 h after adjuvant injection. Saline injection did not increase βPPT-A or α-CGRP mRNA expression in DRG neurones, in fact α-CGRP levels were significantly decreased (figures 3.6 and 3.7).

Over the same time course the proportion of small neurones with clear expression of α-CGRP and βPPT-A mRNAs was also monitored. 30 min after injection no change was seen, however by 1 hour the proportion of expressing neurones had increased significantly ipsilateral to injection. This increased proportion of α-CGRP and βPPT-A expressing neurones was maintained at subsequent time points (figures 3.8 and 3.9).

To determine whether the increase in PPT mRNA is reflected by new transcription, ISH was performed using a probe complementary to intron E of the β-PPT gene. Photomicrographs of this experiment are displayed in figure 3.10 and 3.11. Quantification of silver grains demonstrated that within 30 min of injection of FCA

there was increased hybridisation in small diameter neurones (figures 3.12), correlating with the increases seen in mature mRNA. This suggests that the early increases in mRNA expression are, at least in part, due to increased transcription.

Cycloheximide pre-treatment did not affect the FCA-induced increase in α-CGRP mRNA per small diameter neurone 30 min after injection (figure 3.13), indicating that *de novo* protein synthesis is not a prerequisite for the increase in α-CGRP gene expression. Cycloheximide treatment alone did not cause any changes in α-CGRP encoding mRNA levels over the 90 min experiment.

Figure 3.2 Dark field photomicrograph of PPT mRNA expression in L5 DRG neurones of untreated (control) rats. Original magnification 40x.

Figure 3.3 Dark field photomicrograph of PPT mRNA expression in rat L5 DRG neurones 1h after ipsilateral to injection of FCA around the tibio-tarsal joint. Original magnification 40x. Note the increased density of silver grains per neurone and increased number of expressing neurones compared to figure 3.2.

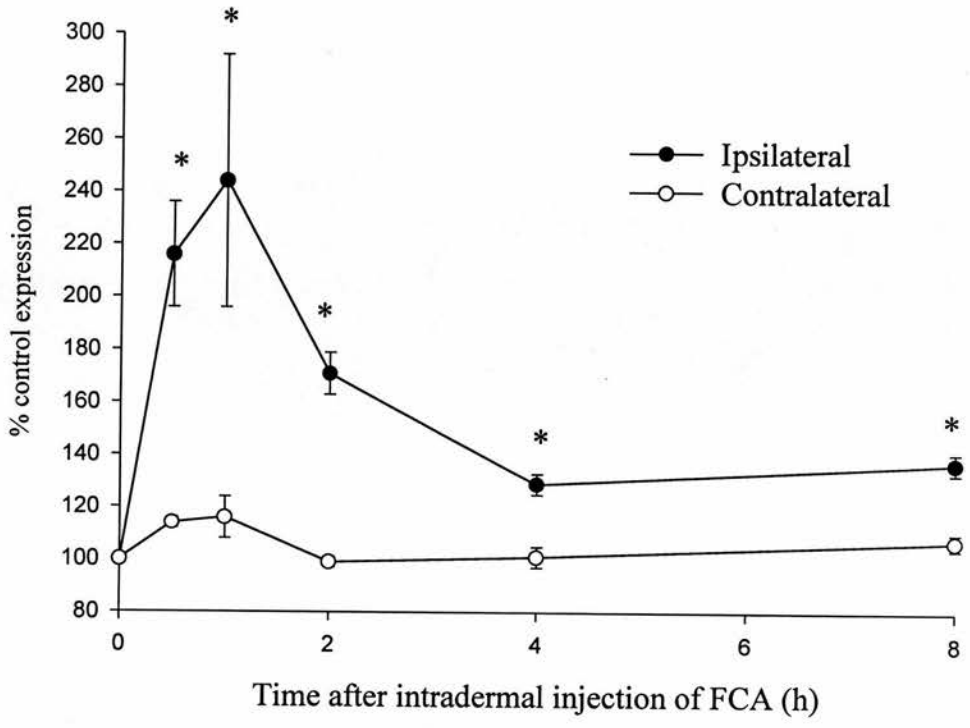


Figure 3.4 Expression of mRNA encoding β-PPTA in small diameter (≤20μm) L5 DRG neurones ipsilateral to intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control. Null hypothesis rejected at $p \leq 0.05$, n=5.

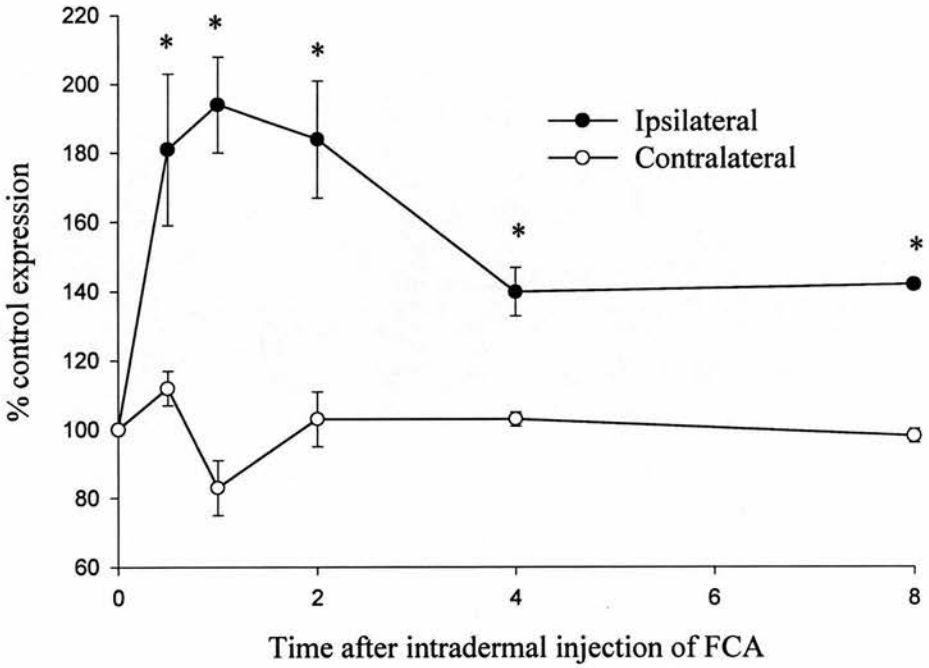


Figure 3.5 Expression of mRNA encoding α-CGRP in small diameter (≤20μm) L5 DRG neurones ipsilateral to intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control. Null hypothesis rejected at $p \leq 0.05$, n=5.

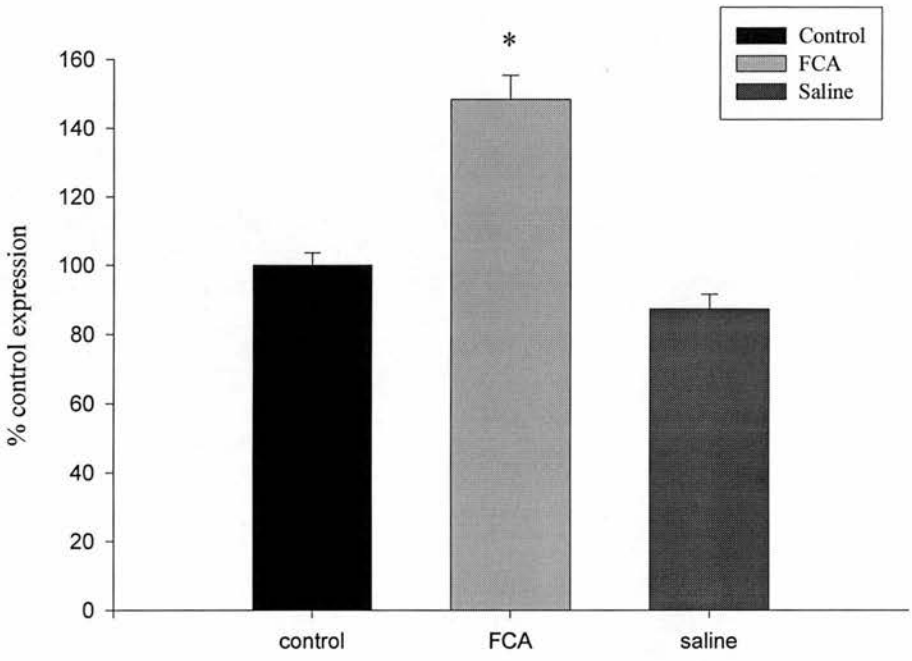


Figure 3.6 Levels of βPPT-A mRNA in innervating L5 DRG 30 min after the injection of saline or FCA around the ipsilateral tibio-tarsal joint. Values (mean±SEM) are expressed as % of untreated controls, n=4-5. *p≤0.05 compared with untreated controls.

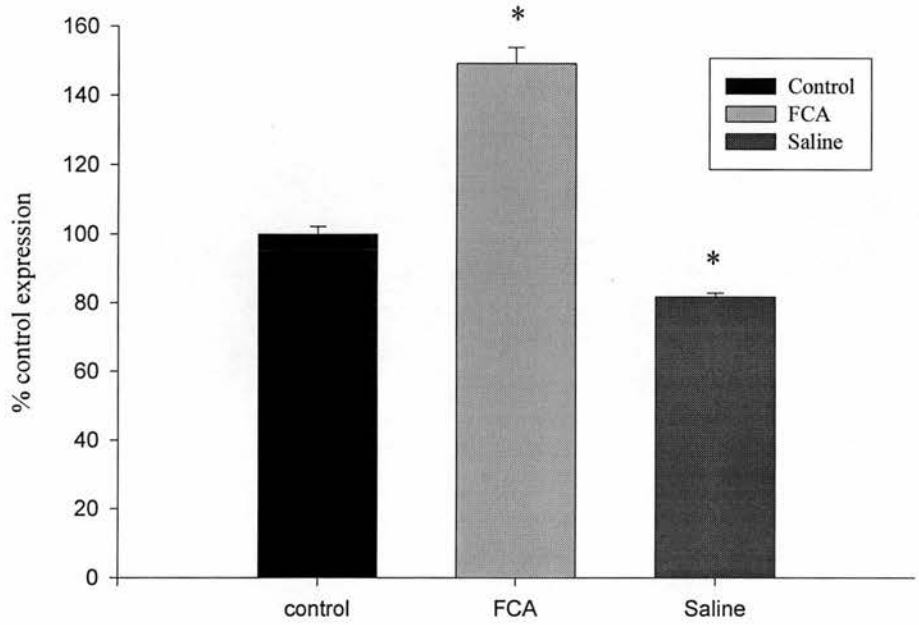


Figure 3.7 Levels of α-CGRP encoding mRNA in innervating L5 DRG 30 min after the injection of saline or FCA around the ipsilateral tibio-tarsal joint. Values (mean±SEM) are expressed as % of untreated controls, n=3-5. *p≤0.05 compared with untreated controls.

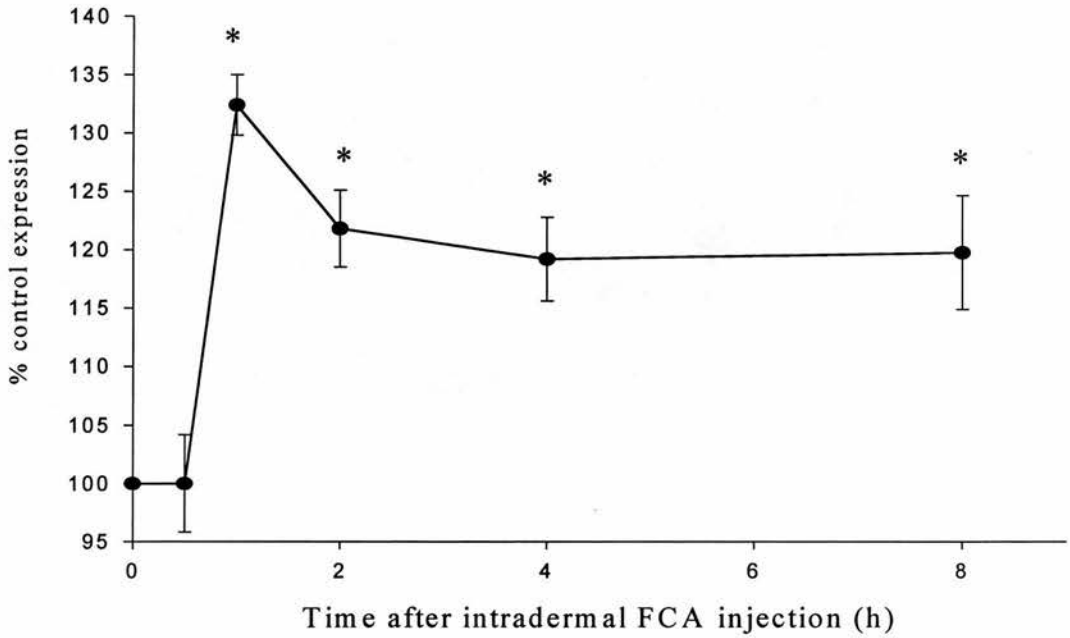


Figure 3.8 Proportion of small diameter (≤20μm) L5 DRG neurones expressing mRNA encoding β-PPTA within 8h of ipsilateral intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control, n=5. *p≤0.05 compared with untreated controls.

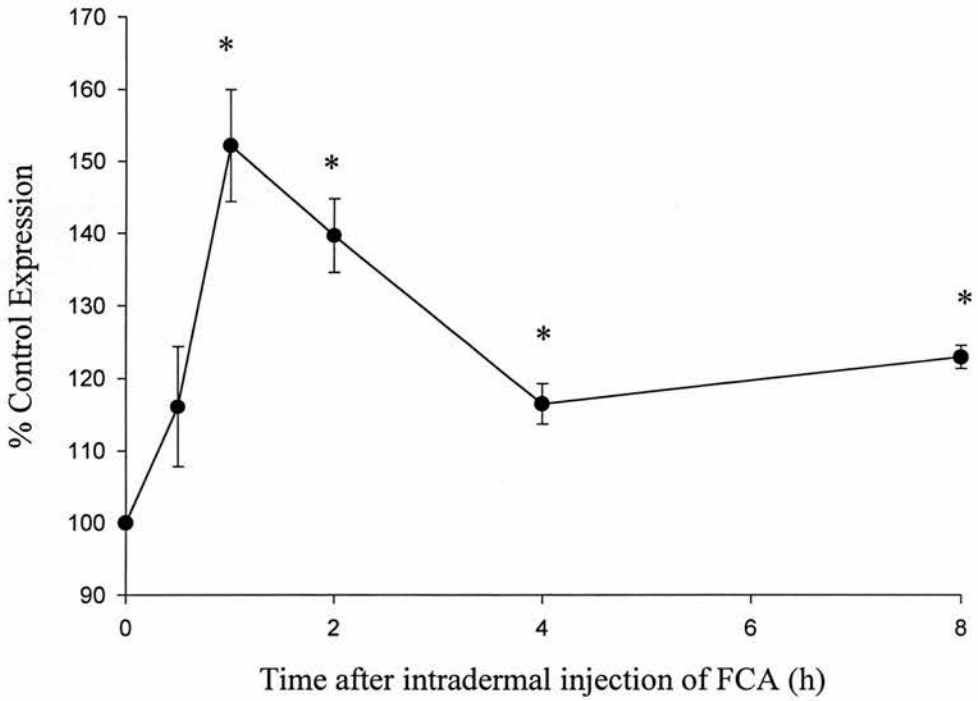


Figure 3.9 Proportion of small diameter (≤20μm) L5 DRG neurones expressing mRNA encoding α-CGRP within 8h of ipsilateral intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control, n=4-5. *p≤0.05 compared with untreated control.

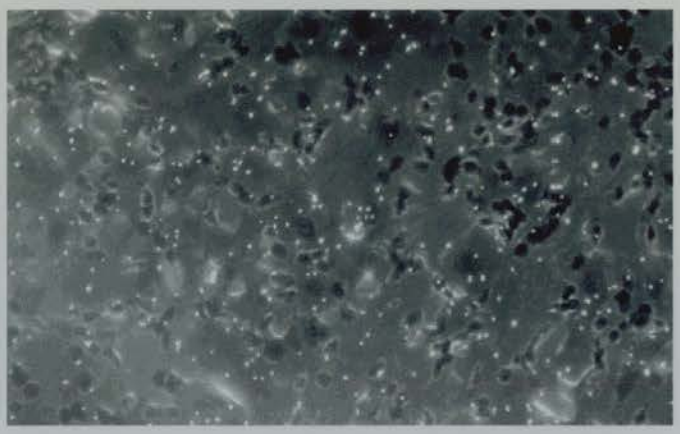


Figure 3.10 Dark field photomicrograph of expression of hnRNA complementary to intron E of βPPT-A in rat L5 DRG neurones of untreated (control) rats. Original magnification 40x.

Figure 3.11 Dark field photomicrograph of expression of hnRNA complementary to intron E of βPPT-A in rat L5 DRG neurones 30 min after ipsilateral injection of FCA around the tibio-tarsal joint. Original magnification 40x. Note increase density of silver grains per expressing neurone.

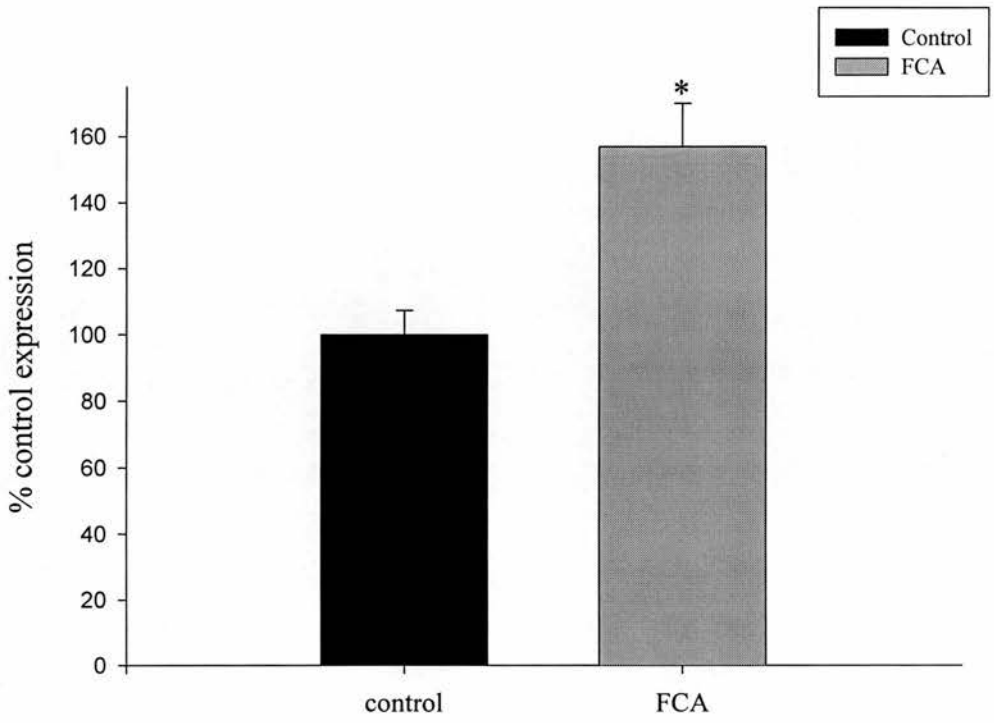


Figure 3.12 Expression of hnRNA encoding intron E of β-PPTA in L5 DRG 30 min after ipsilateral intradermal injection of FCA around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control. n=3-4. *p≤0.05 compared with untreated controls.

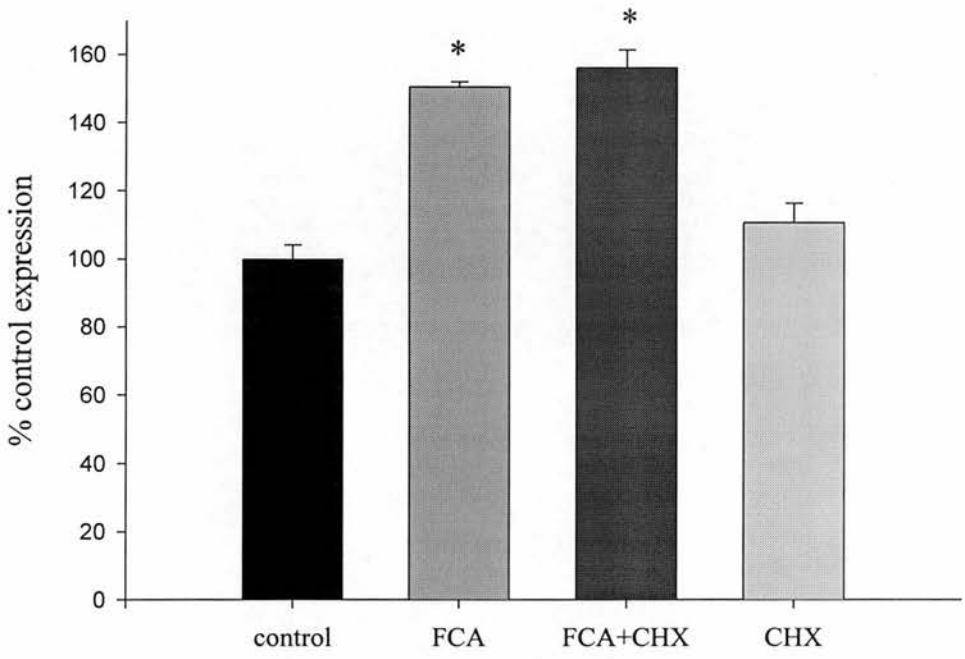


Figure 3.13 Effect of pre-treatment with cycloheximide (3mg/kg ip) on the expression of α-CGRP encoding mRNA in L5 DRG 30 min after ipsilateral intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % of untreated control, n=3-4. *p≤0.05 compared with untreated control.

3.2.2 βPPT-A and α-CGRP mRNA expression in large diameter DRG neurones

The expression of βPPT-A (figure 3.14) and α-CGRP (figure 3.15) encoding mRNAs per large diameter neurone (diameter ≥40μm) after FCA injection initially mirrors that in the small neurones, with a significant increase within 30 min of adjuvant injection, peaking at 1h (180% and 166% of control values for β-PPTA and α-CGRP encoding mRNAs respectively). At subsequent time points levels decrease, so that by 4h levels of both transcripts are similar to untreated controls. Over the same time course there were no observed increases in the proportion of large diameter cells expressing β PPT-A or α-CGRP encoding mRNAs, indicating that there is no recruitment of previously non-expressing neurones (figures 3.16 and 3.17). Indeed, there is a significant decrease in the proportion of large diameter neurones expressing α-CGRP encoding mRNAs 8h after adjuvant injection (figure 3.17).

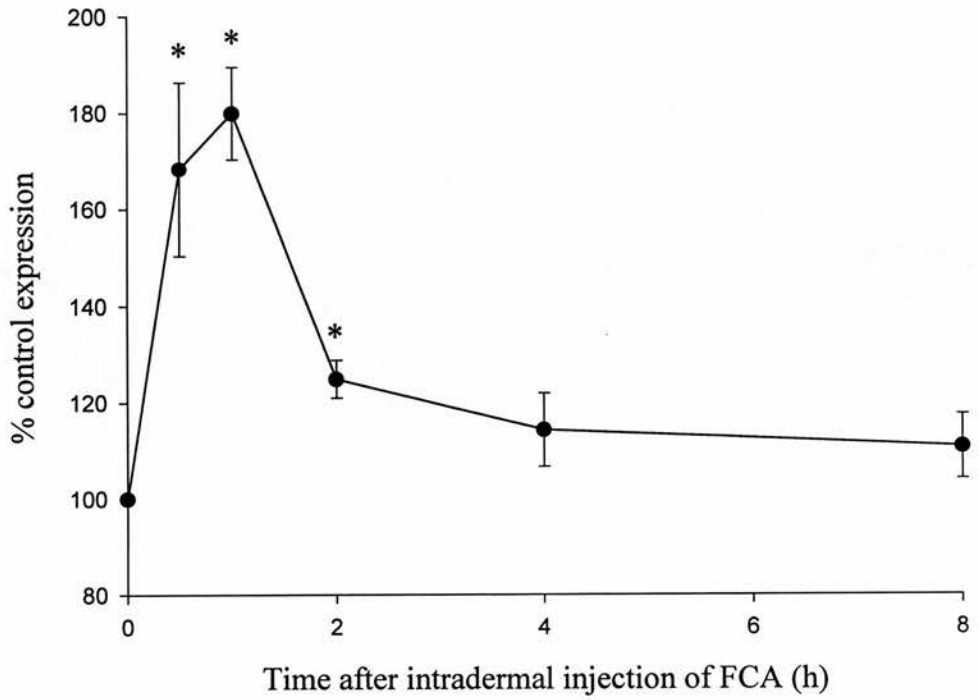


Figure 3.14 Expression of mRNA encoding β-PPTA in large diameter (>40μm) L5 DRG neurones ipsilateral to intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control, n=3-5. *p≤0.05 compared with untreated control.

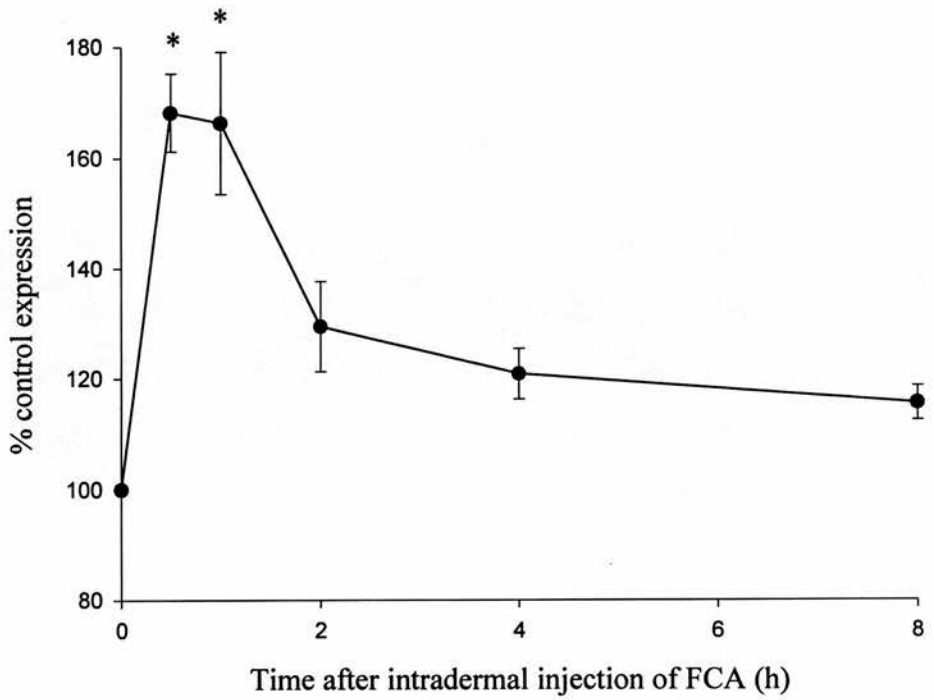


Figure 3.15 Expression of mRNA encoding α-CGRP in large diameter (>40μm) L5 DRG neurones ipsilateral to intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control, n=3-5. *p≤0.05 compared with untreated control.

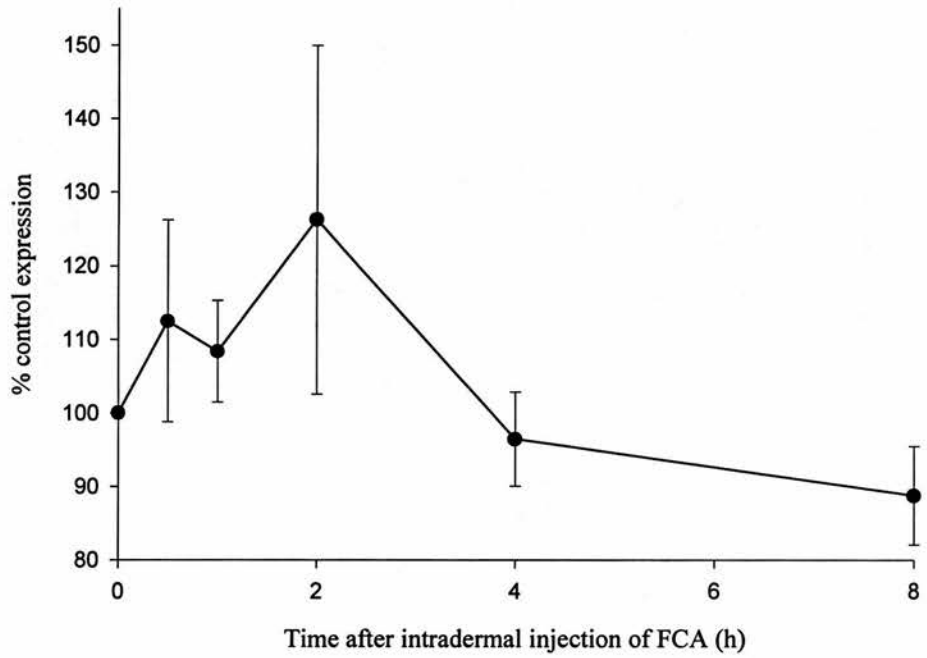


Figure 3.16 Proportion of large diameter (>40μm) L5 DRG neurones expressing βPPT-A mRNA ipsilateral to intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control, n=4-5.

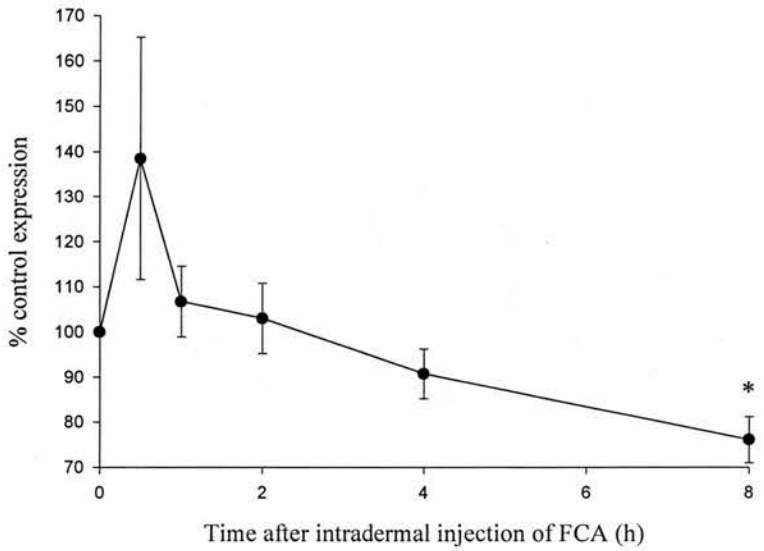


Figure 3.17 Proportion of large diameter (>40μm) L5 DRG neurones expressing mRNA encoding α-CGRP within 8h of ipsilateral intradermal injection of FCA (150μg) around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control, n=3-5.

3.23 Levels of substance P and CGRP in DRG

To explore whether changes in mRNA are reflected in changes in levels of encoded peptide, radioimmunoassay was employed to monitor SP and CGRP levels in innervating DRG following injection of FCA. In line with increases in their encoding mRNAs, levels of both substance P and CGRP immunoreactivity also increased in ipsilateral DRG within 30 min of adjuvant injection (figures 3.18 and 3.19) peaking at 60 min. At subsequent time points, peptide levels decreased and reached control values 4 hours after injection. Indeed, substance P content of DRG from injected animals was significantly lower than in noninjected control animals 8 hours after

adjuvant injection.

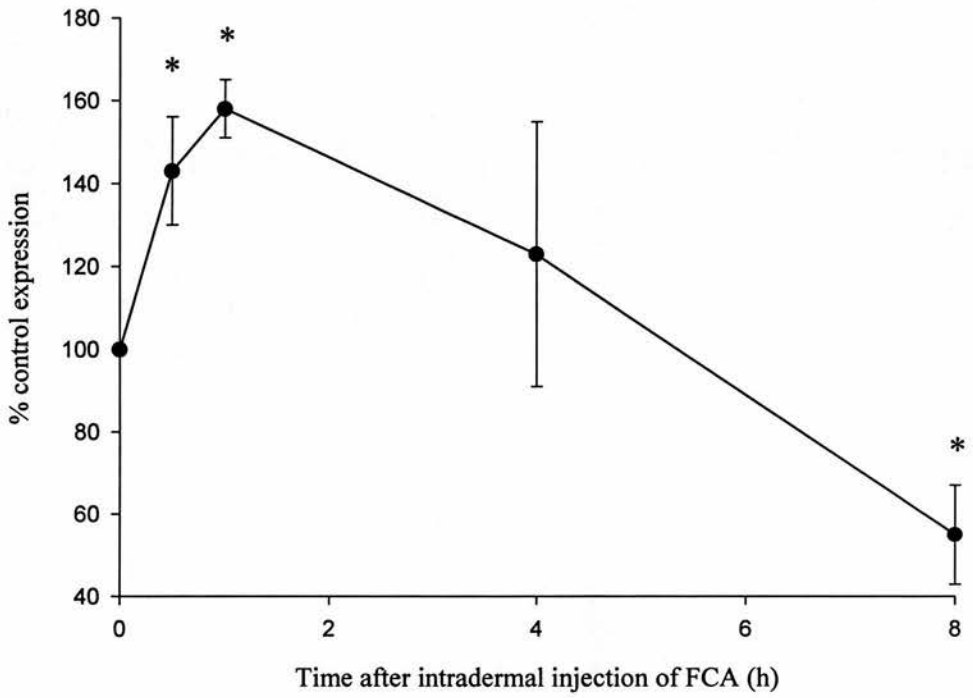


Figure 3.18 Levels of substance P in L5 DRG within 8h of ipsilateral intradermal injection of FCA around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control. n=3-5, *p≤ 0.05, compared with untreated control.

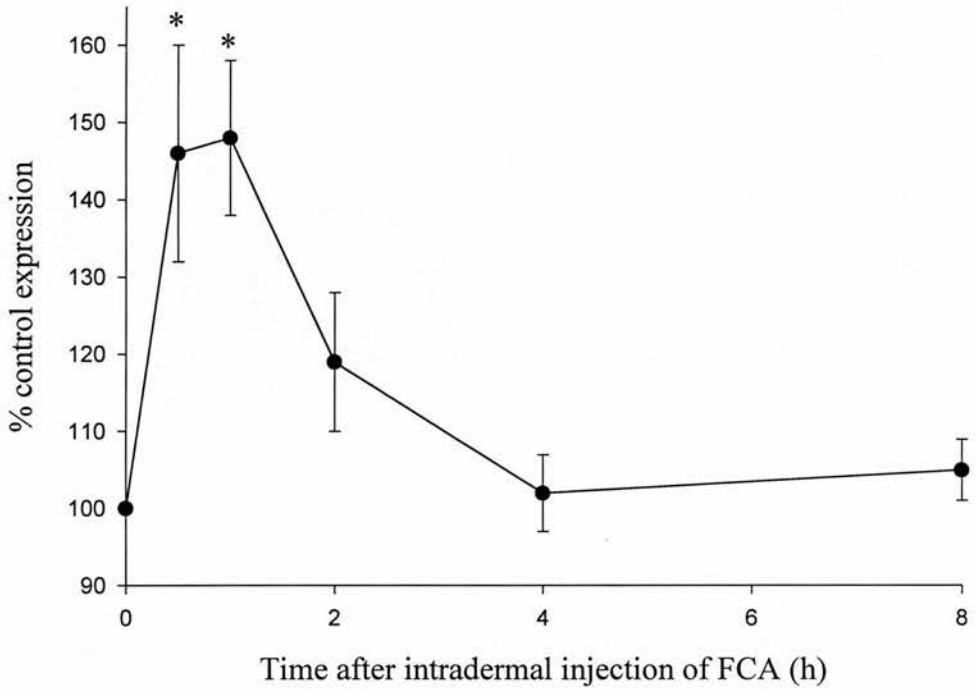


Figure 3.19 Levels of CGRP in L5 DRG within 8h of ipsilateral intradermal injection of FCA around the tibio-tarsal joint. Values (mean±SEM) expressed as % untreated control, n=3-5. *p≤0.05 compared with untreated control.

3.3 Discussion

Data presented here demonstrate a rapid response to FCA injection; 30 min after the injection of adjuvant there is an increase in βPPT-A and α-CGRP encoding mRNAs in both small and large diameter neurones, without an apparent change in the population of expressing cells. These increases are not apparent in L5 DRG of animals injected with saline. By 1h there is an increase in the proportion of small diameter neurones expressing both transcripts, which is maintained up to 8h post FCA injection. No change occurs in the proportion of large diameter neurones

expressing βPPT-A and α-CGRP encoding mRNAs in the first 8h after FCA injection. The increase in α-CGRP encoding mRNAs in L5 small diameter neurones ipsilateral to FCA injection is not prevented by the prior-administration of the protein synthesis inhibitor cycloheximide.

It is possible that the upregulation of neuropeptide gene expression occurs in small and large neurones via a common signalling mechanism. The speed of the increases in neuropeptide mRNAs suggests that they are independent of AP-2, which does not show increases in its encoding mRNA until 1h after FCA injection (Donaldson et al 1995 b). This hypothesis is supported by the observation that cycloheximide pre-treatment does not prevent the increase of α-CGRP encoding transcripts in small diameter neurones 30 min after FCA injection, implying that *de novo* synthesis of AP-2 or any other transcription factor is not necessary in the initial increase in neuropeptide gene expression in response to FCA induced inflammation The increase in sensory neuropeptide mRNAs is augmented by a recruitment of a population of previously non-expressing small diameter cells within the first hour of inflammation. This may reflect the effects of strong transcriptional stimulation in neurones expressing low levels of βPPT-A or α-CGRP encoding transcripts, which were previously below the detection limit of probes used in this in-situ hybridisation. Alternatively, it may signify a change in neuronal phenotype of a population of small diameter cells. In small diameter cells both βPPT-A and α-CGRP have a high basal expression, thus an increase in mRNA expression per neurone coupled with the recruitment of a subpopulation of previously non-expressing cells within 1h of injection signifies a major response to adjuvant injection with a large increase in mRNA available for translation. The high levels of βPPT-A and α-CGRP mRNAs

are significantly elevated in small diameter neurones 8h after injection of FCA, in agreement with previous data using the same model (Donaldson et al 1992).

The mechanism underlying the decrease in the proportion of large diameter neurones expressing α-CGRP mRNA at the 8h time point is unknown. It may represent changes in neuronal phenotype, downregulation caused by an inhibitory feedback mechanism, or neuronal cell death.

The observation that injection of saline around the tibio-tarsal joint does not cause an increase in either βPPT-A or α-CGRP encoding mRNAs implies that the upregulation of these two genes is not due to injection, but attributable to the action of FCA, most probably its immunogenic properties. The mechanism behind the decrease in α-CGRP encoding mRNA after saline injection is unknown, though conceivably may be due to damage to the peripheral nerve caused by the action of injection, a condition which causes downregulation of CGRP expression in innervating DRG (Nahin et al 1994).

It is unclear whether the increased amounts of α-CGRP and βPPT-A mRNAs per small diameter neurone reflect increased gene transcription, or a decrease in the rate of mRNA breakdown by nucleases. This question is partially answered by the results of in-situ hybridisation using a probe complementary to an intronic sequence of PPT-A. The increases in hnRNA transcripts per small diameter neurones 30 min after adjuvant injection can be ascribed directly to increased gene expression. This suggests that the increased hybridisation to the full length βPPT-A probe in small diameter neurones at the same time point are at least in part due to increased transcription of the βPPT-A gene. However this does not eliminate a decrease in mRNA breakdown, and it is conceivable that the increase in total mRNA is due to

both processes.

In other models of joint inflammation increases in the expression of sensory neuropeptides have been noted within 8h of inflammatory stimuli. Bilateral hind limb inflammation induced by intraplantar carrageenan injection caused rapid increases in PPT and CGRP mRNA levels, within 1h of injection, peaking at 2h (Iadarola and Draisci 1988). This was measured by Northern analysis and so represents total mRNA from DRG, and gives no information as to the class of nerve fibre in which these plastic changes occur. A similar argument can be made about experimental data from rats in which RNase protection assays were used to measure neuropeptide gene expression in innervating lumbar DRG after hindpaw formalin injection, which causes an increase in PPT-A mRNA 6h after injection (McCarson and Krause 1995). Using in-situ hybridisation various investigators have reported increases in cell numbers expressing PPT-A encoding mRNA. Noguchi and co-workers described an increase in number of L5 DRG cells expressing PPT-A within 3h and up to 24h after formalin induced inflammation. of the hindpaw, although they observed no changes at 30 min or 1h (Noguchi et al 1988), this may reflect a difference between formalin and FCA induced inflammation. FCA intraplantar injection has previously been reported to cause an increase in the number of ipsilateral DRG neurones expressing PPT-A mRNA within 6h of induction of inflammation (Leslie et al 1995), but neither this or the Noguchi publication detail in which sub-populations of DRG neurones these changes occur. The number of DRG cells expressing PPT mRNA was also increased within 2 days, and persisted up to 20 days after FCA induced inflammation in the rat tibio-tarsal joint (Heppleman et al 1993). A similar stimulus increases the number of CGRP immunoreactive DRG cells

at 2 and 20 days (Hanesch et al 1993).

The increases in βPPT-A and α-CGRP mRNAs are rapidly followed by a swift increase in the encoded peptides, SP and CGRP, within 30 min of FCA injection, levels peaking at 1h. Peptide levels are not elevated above control levels at any time point after 1h despite the elevated levels of their encoding mRNAs. Indeed by 8h SP has decreased to a level significantly below control levels. This is likely to be due to rapid transport of peptides away from the DRG. In inflammation caused by hind-paw injection of heat killed *M. butryricum* there is an increase in immunoreactive CGRP levels in DRG, this increase is augmented by injection of the axonal transport inhibitor colchicine (Kuraishi et al 1989). This suggests that newly synthesised CGRP is transported away from the DRG, presumably to the nerve terminals. In addition axonal transport of CGRP and SP is increased in nerves innervating tissue inflamed by FCA injection (Donnerer et al 1992). Supporting the view that decreases in DRG peptide levels from the peak 1h after adjuvant injection may be attributable to increased axonal peptide transport at a rate which exceeds that of peptide synthesis. One of the enzymes responsible for neuropeptide degradation, neutral endopeptidase (NEP, also called enkephalinase, EC 3.4.24.11), cleaves CGRP at a rate much slower than SP (Katayama et al 1991). This may account for the discrepancy between SP and CGRP levels 8h after FCA injection, where CGRP levels are similar to those found in DRG of untreated animals, but SP are significantly lower than controls.

At time points 1h after FCA injection there is an increase in the number of small diameter neurones expressing βPPT-A and α-CGRP mRNAs. Radioimmunoassays described here were carried out on whole DRG, but it seems likely that this reflects

an increase in the number of small diameter neurones immunopositive for SP or CGRP, mirroring the increase in mRNAs. An increase in the number of small diameter neurones immunopositive for SP or CGRP would effectively increase the number of fibres capable of mediating noxious and painful stimuli. The net effect would be maintenance of increased dorsal horn excitability and augmentation of inflammatory events in the periphery.

There are remarkably similar effects on the expression of βPPT-A and α-CGRP mRNAs and their encoded peptides. Given that there is strong evidence that SP and CGRP are co-localised in a number of sites, including small diameter DRG neurones (Ju et al 1987) it is conceivable that the transcription of both genes are under a common mechanistic control. Co-ordinated expression of the 2 genes would be advantageous given the synergistic action of SP and CGRP both peripherally and centrally (see section 1.6)

As described in chapter 1.2.2, sensory neurones have been classified according to their conduction velocity and the diameter of their cell bodies. In these experiments mRNA expression has been quantified in DRG neurones with diameters ≤20μm and ≥40μm. However there is a degree of overlap in the diameter of the different classes of sensory nerves, for example neurones with a diameter ≤20μm are likely to be either C or Aδ fibres. Although both C and Aδ fibres have been implicated in the transmission of pain, however these classes of sensory nerves have different conduction velocities and innervate different laminae of the dorsal horn of the spinal cord. It is likely that the different classes of nerve fulfil different roles in responses to inflammatory stimuli. In experiments described here it is not clear in which classes of nerve fibres the changes in neuropeptide gene expression occur. The B fragment

of cholera toxin, which binds to GM1 ganglioside, a glycolipid found only in myelinated A type fibres, conjugated to horseradish peroxidase (Robertson et al 1991). GM1 ganglioside conjugated to horseradish peroxidase has been injected sciatic nerve to label myelinated nerves in the innervating DRG (Neumann at al 1996), this approach could be used together with neurone diameter and ISH to elucidate the class of small diameter neurones in which βPPT-A and α-CGRP mRNA levels increase after FCA injection.

As discussed in chapter 1.13 it has been hypothesised that NGF produced in the periphery in response to inflammation is retrogradley transported to the cell bodies of sensory nerves and mediates plastic changes in sensory neuropeptide expression. However, the speed of observed changes in neuropeptide encoding mRNAs in these experiments suggests they are too swift to be dependent upon axonal transport of NGF, measured at 13 mm/h in the rat sciatic nerve (Stockel et al 1975). Indeed the fastest retrograde transport of molecules in the sciatic nerve has been measured at 17mm/h (Ochs 1972), making it unlikely that the initial increases in βPPT-A and α-CGRP encoding mRNAs are mediated by the transport of any molecule from the periphery. Additionally the distribution of high affinity NGF receptor (trkA) in DRG neurones (as discussed in section 1.13) does not exactly match the neurones in which increases in βPPT-A and α-CGRP encoding transcripts are seen, for example trk A mRNA is not co-localised with PPT-A mRNA in large diameter neurones (Kashiba et al 1996). Given the recent discovery that the low affinity neurotrophin factor receptor p75 can activate signalling mechanisms independent of high affinity trk receptors (Carter et al 1996), the distribution of p75, in relation to neuropeptide encoding mRNAs may be of interest. However NGF is extremely unlikely to be

involved in the initial changes in neuropeptide gene expression seen within the first hours but may have a role in modulating neuropeptide gene expression during the latter stages of the inflammatory process.

4 Plasticity of Nervous and Immune Systems During Acute Joint Inflammation

4.1 Introduction

As discussed in chapter 1.2 the nervous system has been implicated in the development of joint inflammation. This view is supported by results presented in chapter 3, where increases in mRNAs of βPPT-A and α-CGRP encoding gene are seen in small diameter DRG neurones (≤20μm), which is the size of nerve associated with transmission of nociceptive information, within 30 min of joint inflammation induced by FCA. Previous experimental data has shown the increases in mRNAs of βPPT-A and α-CGRP encoding gene seen in small diameter L5 DRG neurones within 8h of FCA injection can be prevented by pre-administration of the local anaestheic, lignocaine, around the sciatic nerve (Donaldson et al 1994). Taken together with the observation that application of 40mM K^+ to cultures of adult DRG neurones causes an upregulation of PPT-A expression (Mulderry et al 1993 b) the evidence suggests that neural activity is pivotal to plastic changes in the expression of genes encoding neuropeptides.

It would therefore be relevant to investigate the neural activity of a nerve innervating a joint immediately after FCA injection. The tibio-tarsal joint of the rat receives sensory innervation from the tibial nerve, an anterior branch of the sciatic nerve (Greene 1959). The knee-joint is innervated by an articular branch of the common peroneal nerve, a nerve which is derived from the sciatic nerve. Thus the knee shares a common sensory innervation with the tibio-tarsal joint, with cell bodies located in the same DRG (L4-6) (Greene et al 1959). The effect of injection of FCA (100μg,

intra-articular) into the knee joint on the rate of neural discharge of small diameter innervating sensory nerves was studied.

Blocking propagation of action potentials in the sciatic nerve with lignocaine can be employed to determine whether any changes in neural activity underlie the increases in neuropeptide encoding mRNAs in small diameter DRG neurones innervating a tibio-tarsal joint within 30 min of FCA induced inflammation.

FCA injection and other models of joint inflammation are associated with joint swelling (oedema) and development of hyperalgesia, the exaggerated response to mechanical, chemical and thermal stimuli previously perceived as innocuous (Schaible and Grubb 1993, Donaldson et al 1993). Oedema formation, an early event in inflammation (see chapter 1.6.1), can be measured by a variety of methods, the most convenient being measurement of the joint circumference at various time points after FCA injection. At the same time points development of mechanical hyperalgesia can be measured in terms of pressure required to evoke reflex withdrawal of the ipsilateral limb.

As described in 1.6.1 immune cell infiltration into the inflamed area occurs later in the inflammatory process than oedema formation. This response is seen 14 days after FCA injection into the tibio-tarsal joint (Donaldson et al 1993), a time point at which there are signs of articular cartilage breakdown, proliferation of synovial tissue, pannus formation and bone resorption. The progression of inflammation was followed by monitoring the histological changes in the tibio-tarsal joint over the first 24h of inflammation induced by FCA injection.

4.2 Results

4.2.1 Effect of FCA injection on neural activity

As shown in figure 4.1, FCA injection into the knee joint, as described in 2.6.1 causes an increased frequency in action potentials in innervating fine diameter nerves within 15 min, the discharge seen is similar to that observed after stimulation of polymodal C-fibres by capsaicin, or mechanical stimulation of the joint capsule. This increased activity was still evident 30 min after adjuvant injection, however by 1h activity was not statistically elevated above that recorded from untreated animals.

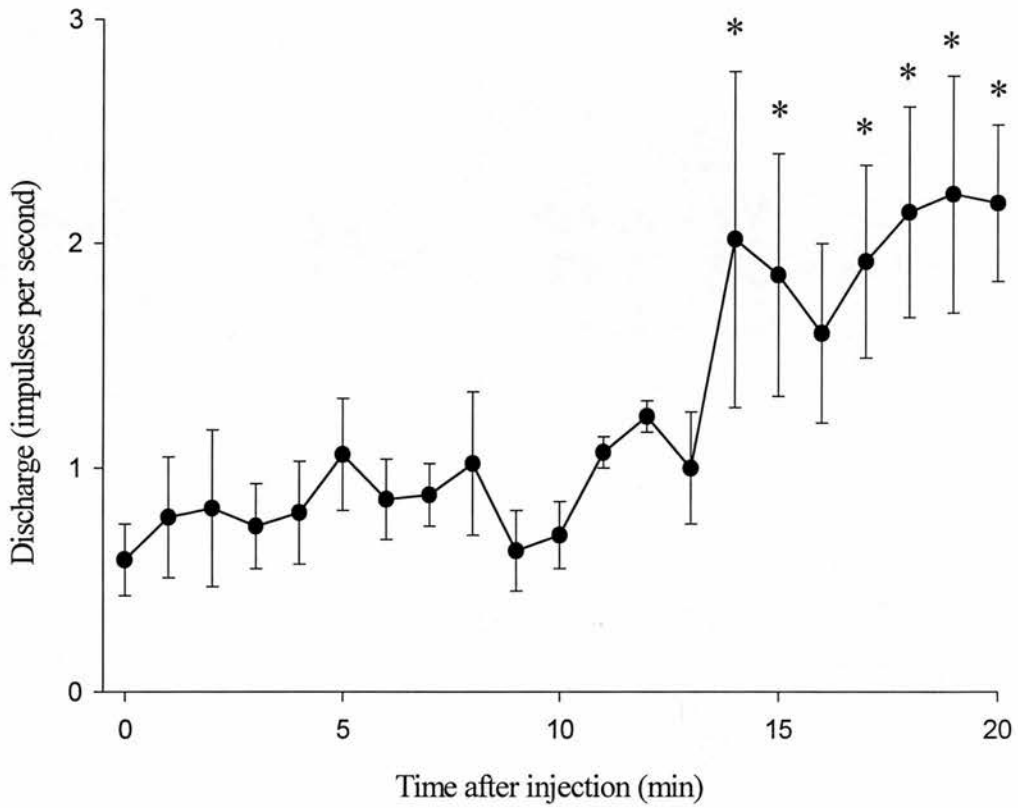


Figure 4.1 Changes in the frequency of action potentials in fine diameter sensory nerves innervating the rat knee joint after FCA injection (100µl i.a.). Action potentials defined as the response of polymodal C fibres to capsaicin and/or mechanical stimulation of the joint capsule. n=5, *p≤0.05 compared with recordings taken from untreated animals prior to FCA injection (0.68±.27). At 30 min discharge is still significantly above levels of untreated control animals (2.34±0.56), at 60 min levels are still elevated but are not statistically different from controls (1.93±.0.51). Values represent single units isolated from multiple unit recordings.

4.2.2 Role of neural activity in plasticity in βPPT-A and α-CGRP gene expression

Inflammation of the tibio-tarsal joint caused by FCA injection, as described in section 2.1, has previously been seen to cause a substantial increase in both βPPT-A and α-CGRP encoding mRNAs in small diameter DRG neurones within 30 min of injection (see section 3.2.1). This effect was reaffirmed by these experiments, however rats showing flaccid paralysis after mid thigh injection of lignocaine to the sciatic nerve, applied without exposure of the nerve (see section 2.6.2), did not display rapid increases in the levels of βPPT-A and α-CGRP encoding mRNAs in response to FCA injection. (figures 4.2 and 4.3)

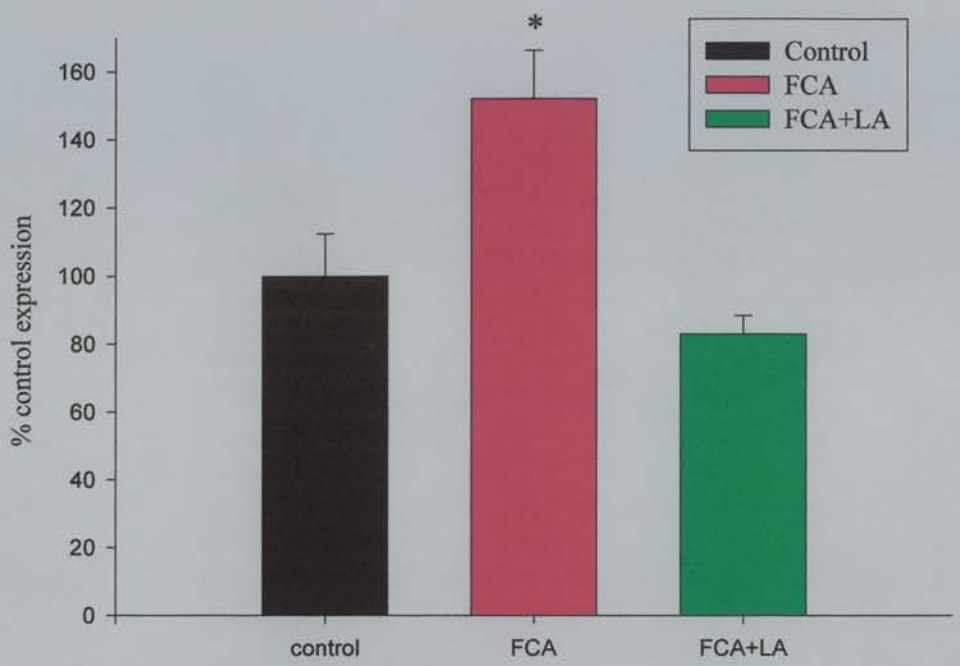


Figure 4.2 Effect of preadministration of lignocaine (2%) around the sciatic nerve on the expression of mRNAs encoding β-PPTA in L5 DRG 30 min after ipsilateral sub-dermal injection of FCA. Values (mean±SEM) expressed as % untreated control, n=3-5 .*$p \leq 0.05$ compared with untreated control.

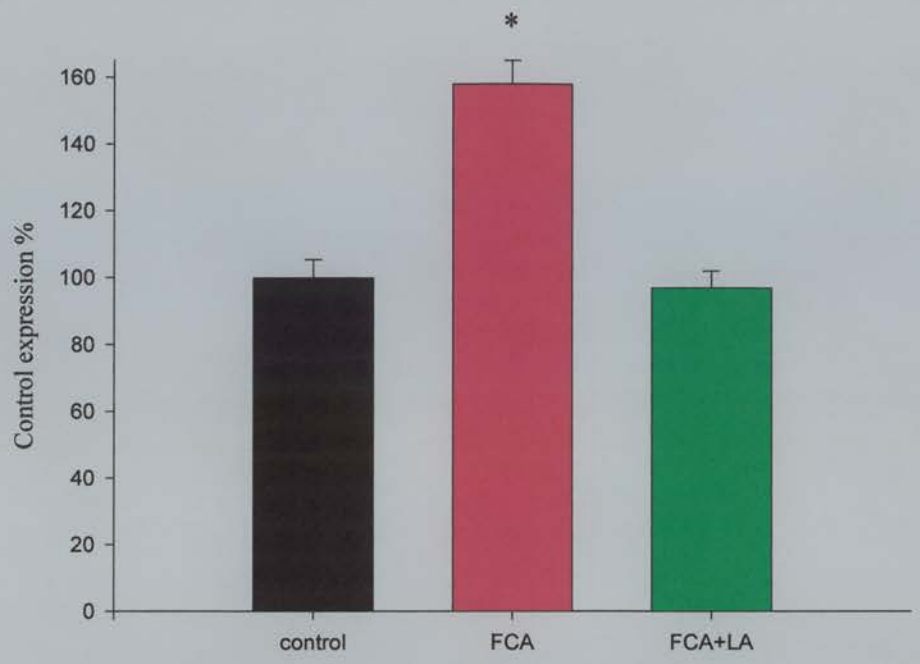


Figure 4.3 Effect of preadministration of lignocaine (2%) around the sciatic nerve on the expression of mRNAs encoding α-CGRP in L5 DRG 30 min after ipsilateral sub-dermal injection of FCA. Values (mean±SEM) expressed as % untreated control, n=3-5. *p≤0.05 compared with untreated control.

4.2.3 Progression of inflammation, assessed as development of oedema and mechanical hyperalgesia

Oedema formation was monitored by measurement of joint circumference over the first 8h of FCA induced inflammation (as described in section 2.1). The circumference of the injected joint increased by a significantly within 30 minutes of adjuvant injection and carried on enlarging, so that by 8h the circumference was 48% greater (figure 4.4). There was also a significant increase in the circumference of the uninjected limb, although at all time points after adjuvant injection the injected limb was always markedly and significantly larger. Uninjected rats, used as an absolute control, also showed an increase in joint circumference with time. The latter two observations may be a result of tissue damage caused by repeated measurements. Mechanical hyperalgeisa, assessed by the pressure applied to the tibio-tarsal joint required to evoke reflex withdrawal of the leg, also showed changes over the first 8h of inflammation. The injected limb was withdrawn at a significantly lower pressure than the noninjected limb at all time points after injection. (figure 4.5).

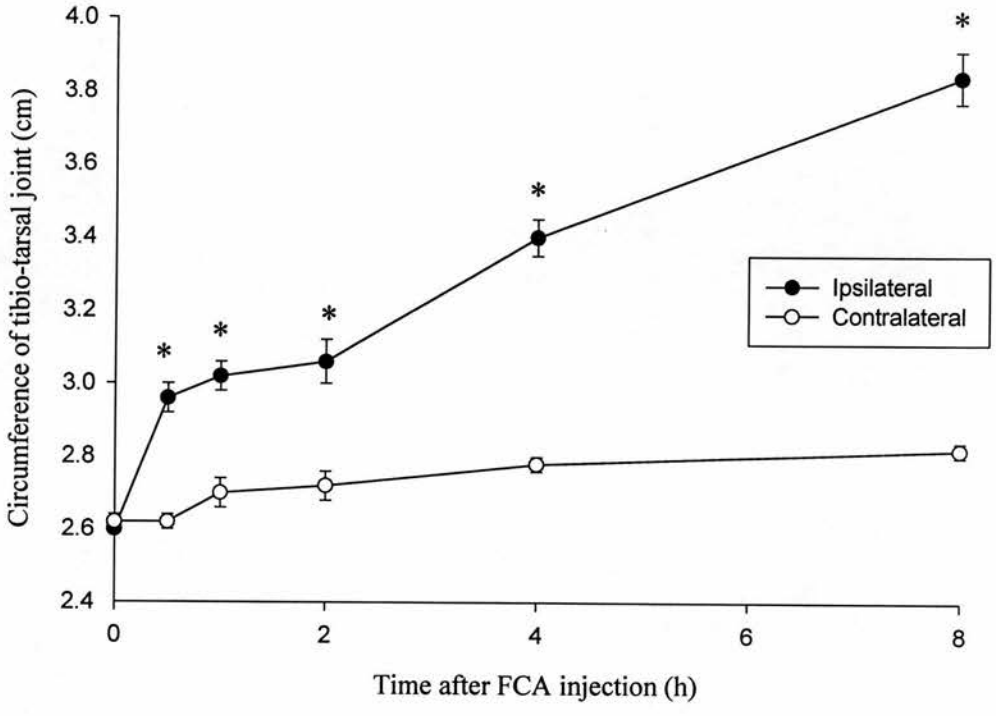


Figure 4.4 Circumference of ipsilateral and contralateral tibio-tarsal joints within 8h of injection of FCA. Values are mean±SEM n=5. *p≤0.05 ipsilateral compared with control at equivalent time points.

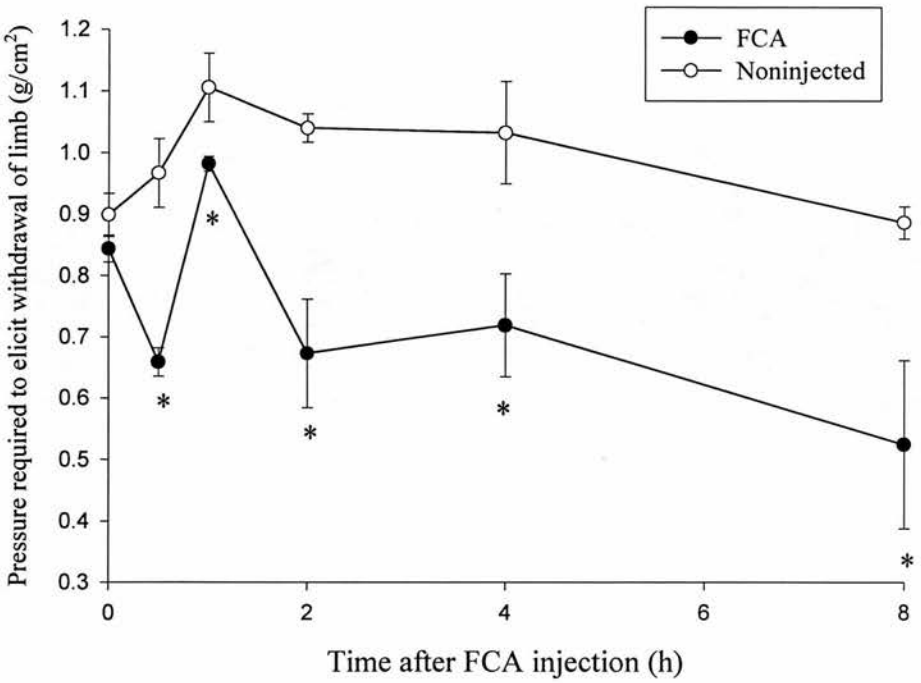


Figure 4.5 Pressure applied to tibio-tarsal joints required to evoke withdrawal of limb in animals with FCA injected tibio-tarsal joint and those receiving no treatment. Values are mean±SEM n=5. *p≤0.05, injected compared with noninjected animals at equivalent time points.

4.2.4 Changes of histology of tibio-tarsal joints after adjuvant injection

The changes in tibio-tarsal joint histology over the first 24h of FCA induced joint inflammation were assessed as described in section 2.10 and shown in figures 4.6-

4.10. Over this time period there were no signs of cartilage or bone breakdown, proliferation of the synovial tissue, or pannus formation. Effects were limited to the periarticular soft tissue surrounding the joint, where inflammatory cell infiltration was noted at 8h (figure 4.8) and 24h (figure 4.9) after ipsilateral FCA injection. No changes in the histology of the tibio-tarsal joint or surrounding soft tissue contralateral to injection were apparent at the 24h time point.

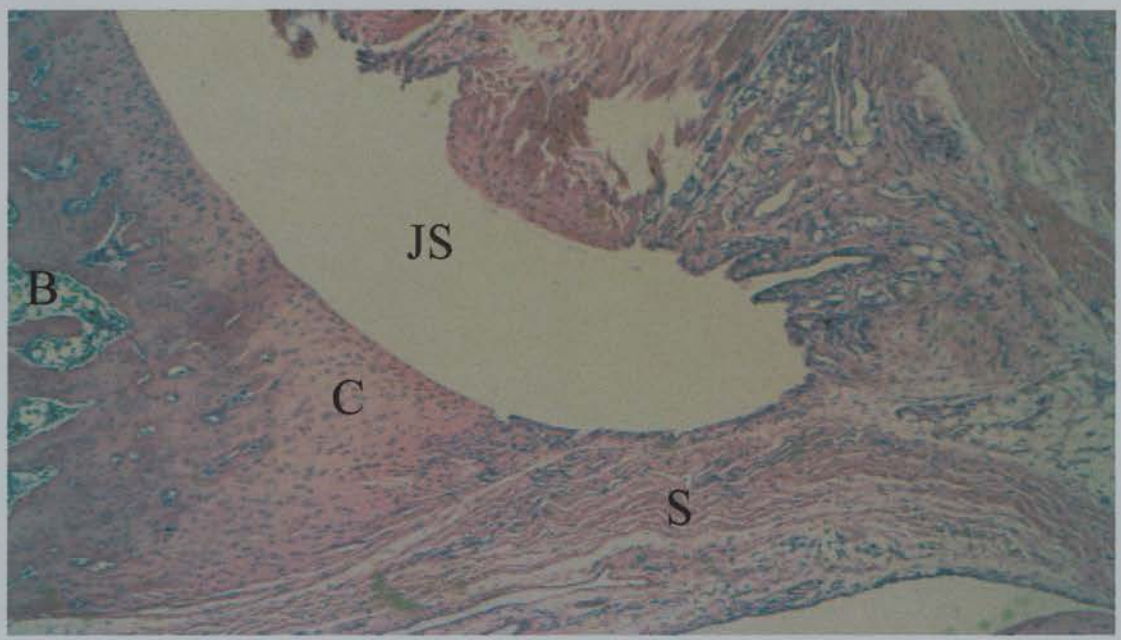


Figure 4.6 Histological apperance of control (uninjected) rat tibio-tarsal joint
JS=joint space, C=cartilage, B=bone, S=synovial tissue

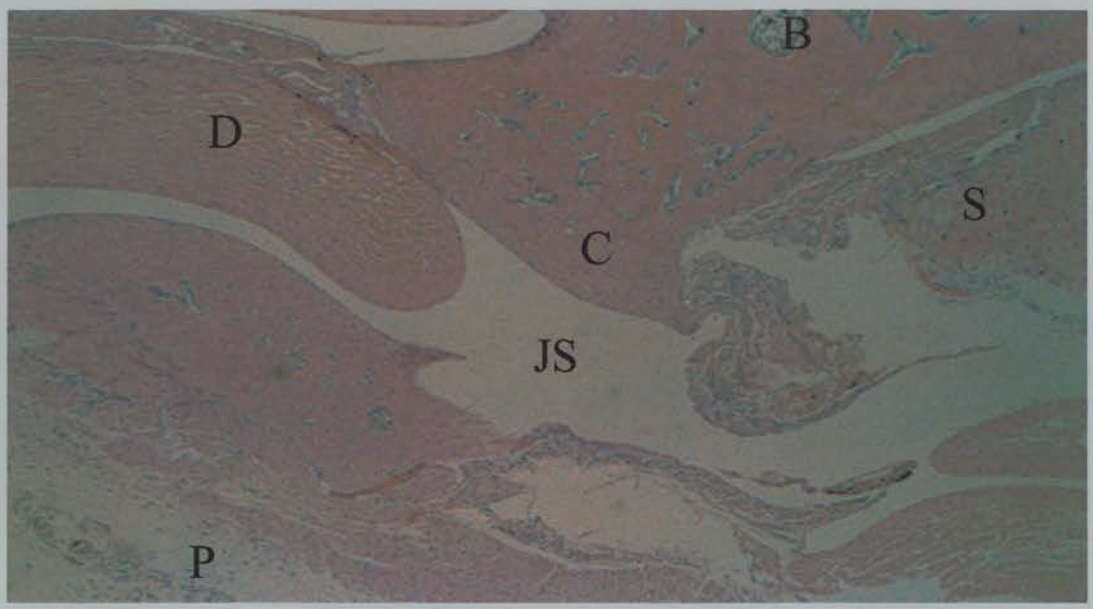


Figure 4.7 Histological apperance of rat tibio-tarsal 1h after ipsilateral injection of FCA, containing 150mg M.tuberculosis, showing no signs of inflammation of the joint or surrounding periarticular soft tissue.
JS=joint space, C=cartilage, B=bone, S=synovial tissue, D=dense connective tissue eg tendon, joint capsule, ligament, P=periarticular soft tissue

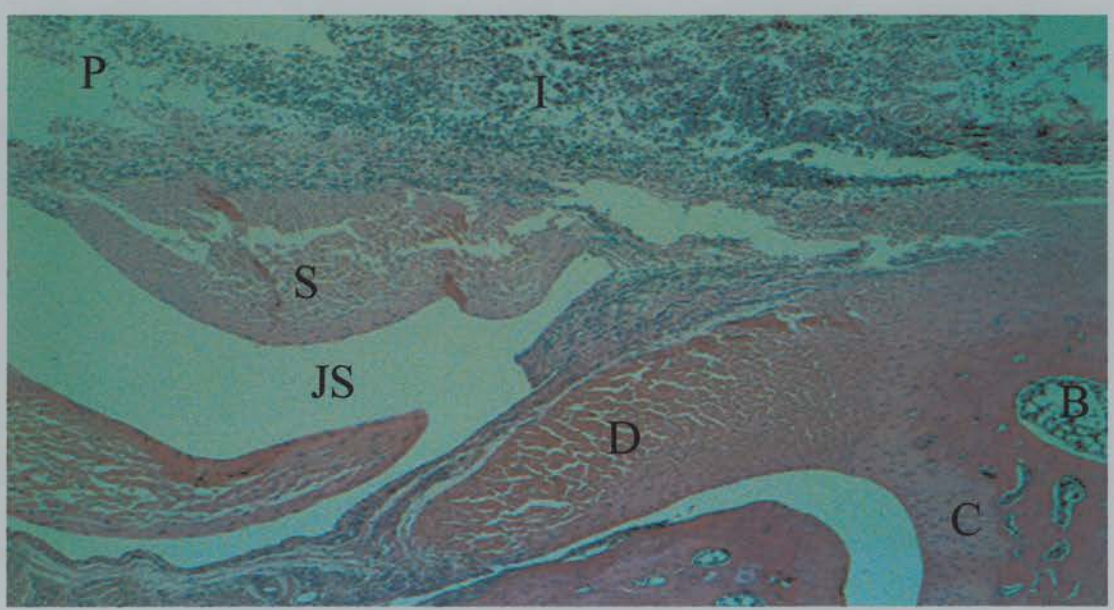


Figure 4.8 Histological apperance of rat tibio-tarsal 8h after ipsilateral injection of FCA, containing 150mg M.tuberculosis, showing inflammatory cell infiltration of periarticular soft tissue, but not the joint.
JS=joint space, C=cartilage, B=bone, S=synovial tissue, D=dense connective tissue eg tendon, joint capsule, ligament, P=periarticular soft tissue, I=inflammatory cell infiltration

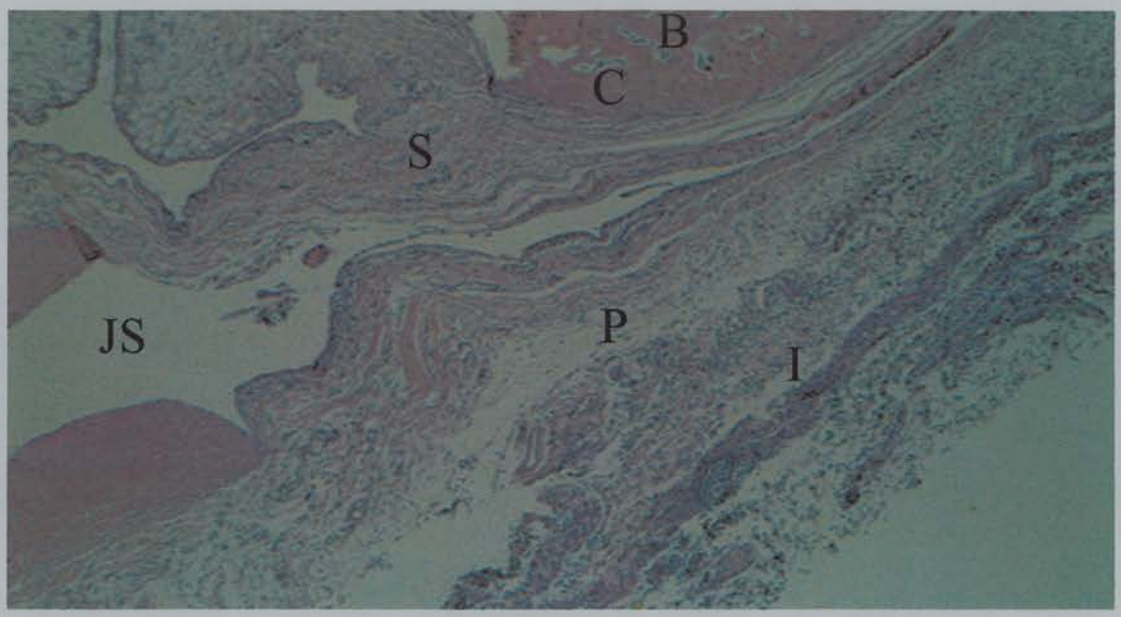


Figure 4.9 Histological apperance of rat tibio-tarsal 24h after ipsilateral injection of FCA, containing 150mg M.tuberculosis, showing inflammatory cell infiltration of periarticular soft tissue, but not the joint.
JS=joint space, C=cartilage, B=bone, S=synovial tissue, P=periarticular soft tissue, I=inflammatory cell infiltration

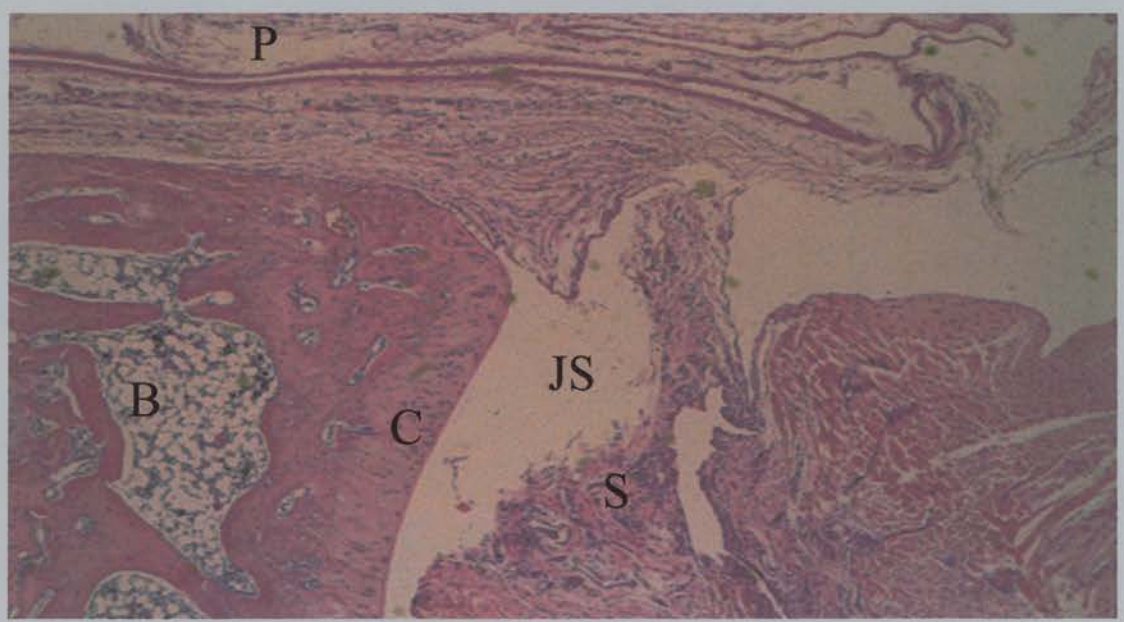


Figure 4.10 Histological apperance of rat tibio-tarsal 24h after contralateral injection of FCA, containing 150mg M.tuberculosis, showing no inflammatory cell infiltration of periarticular soft tissue or the joint.
JS=joint space, C=cartilage, B=bone, S=synovial tissue, P=periarticular soft tissue

4.3 Discussion

The increased frequency of action potentials, which are similar to those observed after capsaicin application to polymodal C-fibres, implicate C-fibres in the early neural response to FCA injection. Changes in neural activity in sensory nerves innervating an inflamed joint have also been detected in the kaolin/carrageenan model of joint inflammation in the cat knee joint. Investigators have reported that 4-5h after injection there is an increase in the basal discharge of group III and IV afferents (C and Aδ fibres), and an increase in the number of units displaying resting discharge (Coggeshall et al 1983).

The expression of mRNAs encoding βPPT-A and α-CGRP in the cell bodies of small diameter nerves, such as C-fibres also increases rapidly (30 min) after FCA injection. This suggests that neural activity may underlie the induction in neuropeptide expression, a hypothesis supported by the prevention of FCA induced increases in mRNAs by blocking of nerve conduction with pre-administration of local anaesthetic. Local anaesthetics block the conduction in small diameter unmyelinated fibres first, and for the longest duration. In these experiments, only animals treated with local anaesthetics which displayed complete motor paralysis were subsequently injected with adjuvant. Thus in these animals the propagation of action potentials in C-fibres, the frequency of which appear to be increased after FCA injection, are definitely blocked as there is no conduction in larger motor neurones. As previously stated depolarisation of cultures of DRG neurones by application of 40mM K^+ causes an increase in PPT-A mRNA expression (Mulderry et al 1993 b). However this stimulus also increases VIP encoding mRNA in cultures, but the expression of

mRNA encoding this neuropeptide is not increased in FCA induced joint inflammation (Donaldson et al 1992). Elsewhere in the nervous system, depolarisation of the hippocampus with the excitotoxin kainate causes an increase in PPT-A expression *in vivo* (Brene et al 1992).

It should also be noted that local anaesthetics, such as lignocaine, also block axonal transport, but the increases in βPPT-A and α-CGRP encoding mRNAs seen within 30 min of FCA injection are too swift to be reliant upon transport of substances from the periphery, since the maximum rate of axonal transport in the rat sciatic nerve has been measured at 17mm/h (Ochs 1972).

Inflammatory events, as assessed by development of oedema and mechanical hyperalgesia, occur very rapidly after FCA injection and continue in a progressive manner. The significant increases in the circumference of joints contralateral to injection were also seen in uninjected animals, and may reflect local tissue damage due to repeated measurements. The exaggerated response to mechanical stimuli will cause a guarding of the limb, thus protecting the inflamed area from further damaging stimuli.

In other models of joint and peripheral inflammation the two inflammatory parameters of oedema formation and development of mechanical hyperalgesia have also been noted to increase rapidly after induction of inflammation. Intraplantar hindpaw injection of FCA, which like FCA injection around the tibio-tarsal joint causes increases in PPT-A mRNA in innervating DRG within 6h (Leslie et al 1995), has been shown to result in oedema formation and increased mechanical and thermal hyperalgesia within 6h (Stucky et al 1993, Galeazza et al 1995, Safieh-Garabedian et al 1995). Marked oedema of the paw occurs within 15 min of hindpaw inflammation

due to injection of carrageenan as measured by paw weight (Gilligan et al 1994), whilst the same stimulus, but at a higher dose, causes oedema formation within 4h as measured by paw width (Iadarola and Draisci 1988).

The formation of oedema occurs at, or near, the site of injection, suggesting it may be a consequence of the release of local inflammatory mediators. This hypothesis is supported by the observation that rats lacking central innervation, due to sectioning of the sciatic nerve, develop oedema similar to that seen in non-transected rats in response to injection of the tibio-tarsal joint with *M. butyricum* (Ahmed et al 1995 b). A number of these inflammatory mediators have also been proposed to sensitise peripheral nerve terminals (see chapter 1.4), contributing to primary hyperalgesia. Indeed sensitisation of group II, III and IV afferents (Aβ, Aδ and C fibres respectively), in response to mechanical stimuli including previously unresponsive group III and IV afferents has been reported within the first hour of joint inflammation caused by injection of kaolin/carrageenan into the knee joint of the cat, with further sensitisation 2-4h after injection (Schaible and Schmidt 1988).

It is unclear if the observed mechanical hyperalgesia involves secondary hyperalgesia, due to sensitisation of the dorsal horn to peripheral input, a process which is postulated to be mediated by CGRP and the tachykinins, SP and NKA (see chapter 1.6.2) Depolarisation of peripheral sensory nerves by electrical stimulation leads to SP and CGRP release into the dorsal horn of the spinal cord (Yaksh et al 1980, Klein et al 1990). Here it has been shown that FCA injection causes an increase in action potentials in small diameter innervating nerves, most probably C-fibres, which widely express SP, NKA and CGRP. Thus it is probable these neuropeptides are released into the dorsal horn of the spinal cord very soon after

FCA injection. Indeed in studies using intraplantar FCA injection, it has been postulated that SP may be released into the dorsal horn of the rat within 6h of inflammatory stimulus (Stucky et al 1993). However the origin of the SP released is unclear; it may be derived from primary afferents, or neurones of descending or intrinsic origin. The same applies to increases of SP release from slices of the spinal dorsal horn taken from rats 3h after hindpaw carrageenan injection. However the release of CGRP, which is solely derived from primary afferents also increased (Garry et al 1992).

Elevated levels of NKA are detectable in dorsal horn of the cat spinal cord immediately after inflammation induced by injection of kaolin and carrageenan into the knee joint (Hope et al 1990). In the same model there is an increase in CGRP release into the spinal cord and enhanced CGRP release in response to both innocuous and noxious stimuli within the first hours of inflammation; the latter effect is also seen in rats (Schaible et al 1994). Furthermore there is evidence of secondary hyperalgesia in response to thermal stimuli in this model, with a decrease in paw withdrawal latency in response to thermal stimuli within 4h and up to 24h after kaolin/carrageenan injection into the rat knee joint (Sluka and Westlund 1993).

SP and NKA have been implicated in mediating vasodilation and increased vascular permeability at the site of inflammation (see chapter 1.6.1), events which underlie oedema formation. However mice lacking SP and NKA, due to a disrupted PPT-A gene, show the same degree of oedema formation as wild type mice in the hindpaw 1 and 3 days after FCA injection (Cao et al 1988). This suggests oedema formation can occur without SP or NKA, but it should be noted that these animals have normal levels of CGRP, which is proposed to be the primary mediator of vasodilation, whilst

other inflammatory mediators, such as bradykinin, histamine and 5-HT are known increase vasodilation and permeability of the microvasculature (Rang and Dale 1991). PPT-A knockout mice also display increased thresholds to moderate and intense noxious thermal and mechanical stimuli. This evidence implicates SP and NKA in the transmission of specific window of stimuli, consistent with their high level of distribution in small diameter nerve fibres associated with transmission of high threshold stimuli.

In previous experiments performed in this laboratory the histology of tibio-tarsal joints 14 days after injection of FCA containing 150μg *M.tuberculosis* showed infiltration of the joint by inflammatory cells, synovial hyperplasia and oedema, pannus formation and subchondral bone resorption. The development of these pathological features was found to correlate with the increase in joint circumference and mechanical hyperalgesia (Donaldson et al 1993). At time points up to 24h after adjuvant injection none of these histopathological changes are apparent in the present study. After 8h, infiltration of the periarticular soft tissue by inflammatory cells is visible. At 24h the cellular infiltration is still limited to the soft tissue surrounding the joint, suggesting that inflammation is limited to the site of injection up to 24h after FCA administration. In agreement with the phases of inflammation discussed in chapter 1.6.1, oedema formation and development of mechanical hyperalgesia are apparent at time points before cellular infiltration. As discussed in section 1.6.1.4 SP, NKA and CGRP have a number of chemotactic and proliferative effects on immune cells, thus the release of these neuropeptides from sensory nerves at or near the site of injection may contribute to the cellular infiltration noted after 8h.

These data indicate that changes observed in neuropeptide encoding gene expression

after FCA injection are not dependent upon changes in the tibio-tarsal joint, thus at this acute stage the model is better described as one of peripheral inflammation rather than joint inflammation.

The maximum rate of axonal transport in the sciatic nerve has been measured at 17mm/h (Ochs 1972), most molecules are transported at a slower rate, including SP which has been measured at 5-10mm/h (Brimijoin et al 1980, Harmar and Keen 1982). This rate of axonal transport dictates that newly synthesised SP will not reach peripheral nerve terminals until very late time points in these experiments. However newly synthesised SP will reach central sensory nerves at much earlier time points. Given the proposed role of these three neuropeptides in both central and peripheral processes underlying inflammation, the upregulation in their production will lead to the augmentation of the inflammatory response to FCA application.

5 Molecular Mechanisms Underlying Neuropeptide Gene Expression

5.1 Introduction

The levels of sensory neuropeptides at nerve terminals are dependent upon the rates of production, as well as axonal transport and breakdown by peptidases. Thus the control of gene expression of neuropeptides in DRG of sensory nerves associated with transmission of nociceptive information innervating inflamed and non-inflamed joints is of considerable interest and relevance. In this chapter the molecular mechanisms underlying the increased expression of the genes encoding βPPT-A and the α-CGRP in L5 DRG following FCA induced joint inflammation are investigated.

A preliminary study has been carried out utilising antisense technology, with the ultimate aim of using antisense oligonucleotides (AS-ON) *in vivo* to block the increases in βPPT-A and α-CGRP expression in DRG and test their requirement in the subsequent development of FCA induced joint inflammation. This preliminary study examined the ability of a number of AS-ON to decrease CGRP and SP expression in primary cultures of adult rat dorsal root ganglion neurones. Cultures of adult rat dorsal root ganglia neurones have been shown to be independent of NGF for survival. Application of NGF to cultures of adult rat dorsal root ganglia neurones induces the expression of PPT-A and α-CGRP (Lindsay and Harmar 1989).

The results obtained from these experiments are detailed below, however they did raise the possibility of the involvement of stress-activated transcription factors in the

increases in SP and CGRP. I have previously shown increases in neuropeptide encoding mRNAs occur very rapidly, within 30 min, of FCA injection. These increases are not prevented by prior treatment with cycloheximide and so are unlikely to require protein synthesis. This would seem to preclude the involvement of immediate early genes in the increases in βPPT-A and α-CGRP gene expression. Similarly, the rapid time course of these increases suggest the involvement of post-translational modification of pre-existing transcription factors. I have concentrated investigations on 2 families of transcription factors, the Rel, including NFκB, and STAT families. Members of both these families are expressed in nervous tissue, are modified by post-translational modification and are implicated in modulating stressful stimuli. These families are discussed in chapter 1.15 and 1.16.

The breakdown of IκB, as an indicator of NFκB activation, in DRG was studied with a view of a possible involvement of NF-κB in the upregulation of the expression of these two neuropeptide encoding genes.

5.1.1 Use of antisense oligonucleotides to specifically inhibit gene expression

Antisense oligonucleotides (AS-ON) are short synthetic single stranded nucleotide sequences (<20 bases), which block synthesis of specific proteins, reviewed in Hunter et al 1995. The AS-ON can be made up of bases found in RNA or DNA. AS-ON are readily taken up into cells by an, as yet, unknown mechanism, despite their large size (for example 17 bp AS-ONs used in these experiments have molecular weights between 5 and 5.5 kDa) and negative charge. Once the AS-ON has entered the cell it is predicted to bind to the target mRNA (or hnRNA), forming a double-stranded nucleic acid species and subsequently blocking protein synthesis. The

mechanisms for this are unclear, but are speculated to be due to:

1)Recognition and degradation of the double stranded nucleic acid by RNase H, an endogenous nuclease which may normally serve a protective role in infection by selectively degrading viral dsRNA.

and/or 2)Prevention of the movement of the ribosome along the mRNA, causing blockage of translation

Based on previous experiments there are a number of basic rules to follow in the design of AS-ON to ensure efficacy.

i)Choice of Nucleotide

The naturally occurring nucleotide bases have been used effectively in antisense oligonucleotides, however their phosphodiester backbone is susceptible to degradation by endogenous cellular enzymes, notably RNase H. This cellular breakdown reduces their life span and thus their efficacy. By modifying the nucleotide it is possible to make more stable backbones. The most stable modifications have been achieved using phosphorothioate nucleotides.

ii) Length

The oligonucleotide must be small enough to be readily taken up by the cell, but must be of a sufficient length to attain specific sequence interaction. A length of 15-20 bases has been found to be optimal.

iii) G:C Content and Sequence Motifs to Avoid

Oligonucleotides with a high G:C content have been found to be 'sticky', binding to RNA in a non-specific fashion. However if G:C content is too low the affinity of the oligonucleotide is reduced. Sequences with a GCT core and GGGG motifs should be avoided, as they are known to cause non-specific interactions (Hunter et al 1995).

iv)Oligonucleotide Target

In general, most success has been obtained when the sequence around the translational start point (ATG) of the gene has been selected as the target. Alternatively intron/exon boundaries have proved to be a fruitful target.

5.1.2 Transcription factors and the expression of neuropeptides

In chapter 3 I demonstrated that within 30 min of FCA injection expression of the genes encoding SP and NKA, and CGRP is increased by a mechanism which is independent of *de-novo* protein synthesis. Previous studies have shown that PPT-A expression can be regulated by a wide variety of stimuli (see section 1.8), it has been suggested that these stimuli may act at the level of transcription (Paterson et al 1995 a).

As described in section 1.8, a number of sites capable of binding transcription factors have been identified in areas of PPT-A which can direct expression of a reporter gene micro-injected into primary cultures of DRG neurones. Located in this region of the rat PPT-A gene are sites which interact with AP-1 (Paterson et al 1995a, Morrison et al 1995), octamer binding protein (Mendelson et al 1998), members of the bHLH (E box) family (Mendelson and Quinn 1995, Paterson et al 1995 a,b and c) and the Sp 1 family (Quinn et al 1995, Mendelson et al 1998). Interestingly within the enhancer regions active binding sites for octamer binding proteins and members of the Sp 1 and NFκB families have been identified (Quinn et al 1995, Fiskerstrand et al 1997, Mendelson et al 1998).

The immediate early genes from the Fos/ Jun family are unlikely to be involved in the observed increases in βPPT-A and α-CGRP mRNAs. Members of the Fos/Jun

family are either not expressed DRG neurones, or show no changes in mRNA levels after FCA injection (Donaldson et al 1995 b). The time course of the increases in neuropeptide expression is too rapid to be dependent on *de novo* protein synthesis (a hypothesis supported by experiments involving the inhibitor of protein synthesis, cycloheximide, presented in Chapter 3). In the case of the other proposed modulators of PPT-A expression detailed above, although not immediate early genes, they are not known to be rapidly modified post-translationally following activation of intracellular signalling pathways by stressful stimuli. Therefore it is unlikely that they are involved in the observed increases in either βPPT-A or α-CGRP mRNAs.

A number of transcription factors are known to be held as complexes in the cytoplasm, where they cannot modulate gene expression. Phosphorylation of the cytoplasmic transcription factor complex causes a conformational change revealing a nuclear localisation signal. This directs translocation of the transcription factor to the nucleus, where it can bind at specific DNA motifs and modulate gene expression. Transcription factors of this class are reviewed by Jans (1995) and include the nuclear factor κ B (NFκB) family and signal transducers and activators of transcription (STAT).

5.2 Results

5.2.1 Effect of application of AS-ON to sensory nerve cell culture to levels of SP and CGRP

Following the design criteria outlined in section 5.1.1 phosphorothioate oligonucleotides complementary to short regions of sequences of β-PPTA and α-

CGRP mRNAs were synthesised (Oswel UK). These sequences are described in Table 5.1.

PPT ATG	5' ATT TTC ATG TTG GAT TT 3'
PPT Exon 2	5' CAA TAA TTT AGA TCA TC 3'
CGRP ATG	5' TTC AGA AAG CCC ATG AT 3'
Scramble	5' GTT ATC TTT GTA TGT AT 3'

Table 5.1 Base sequences of phosphorothioate antisense oligonucleotide applied to cultures of adult dorsal root ganglia

Primary cultures of DRG neurones were treated with AS-ONs at two doses, 500nm and 2.5μm. Scramble sequences were used to examine the number of non-specific effects associated with AS-ON. They contain a similar proportion of constituent bases as targeted oligonucleotides, but in a randomised/scrambled order, so will not hybridise with the gene of interest. The CGRP ATG AS-ON acts as an additional scramble when measuring SP, and likewise the two PPT related oligonucleotides act as scramble sequences when measuring CGRP. The results of AS-ON application on CGRP levels in cultures of DRG neurones are illustrated in figure 5.1. Contrary to expectations, all the oligonucleotides at the higher dose increased CGRP production by DRG neurones with a similar magnitude of effect. At the lower dose, only the CGRP ATG AS-ON and scramble AS-ON failed to increase CGRP production.

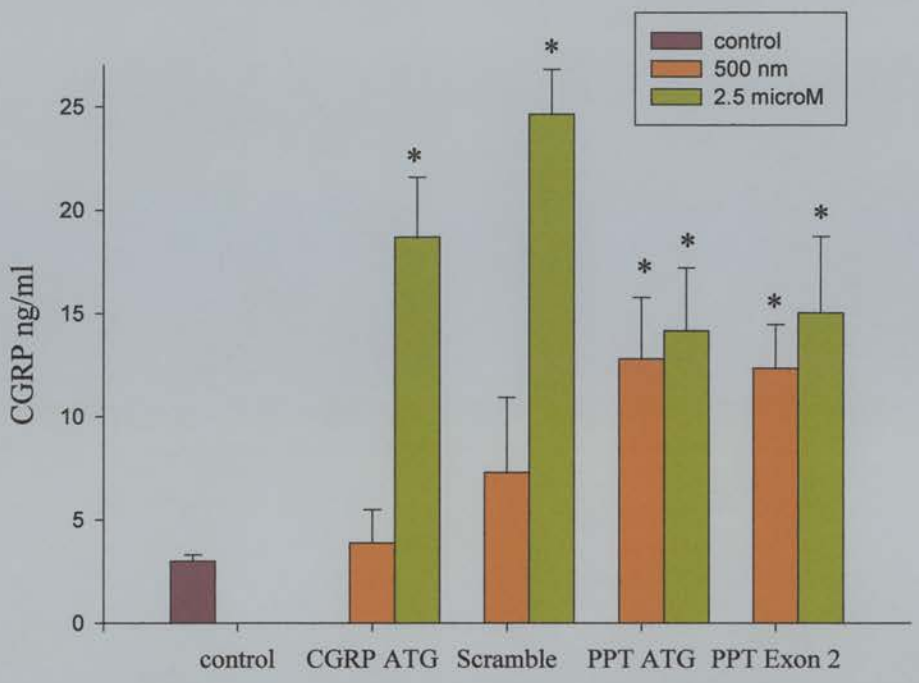


Figure 5.1 Effect of application of phosphorothioate antisense oligonucleotides, at different doses on concentration of CGRP found in cultures of adult DRG neurons. Controls represent levels in untreated cultures. Values are mean±SEM, n=4. Data analysed by ANOVA followed by Dunnett's post hoc test. The Null hypothesis was rejected at $P \leq 0.05$.

The effect of AS-ON application on SP production in cultures of DRG neurones is illustrated in figure 5.2. Statistical analysis of this data was not possible as levels of SP in some samples were below the detection limit for the SP RIA (400 pg/ml). However there was a dose dependent trend, where higher levels of SP were found in cultures treated with higher concentrations.

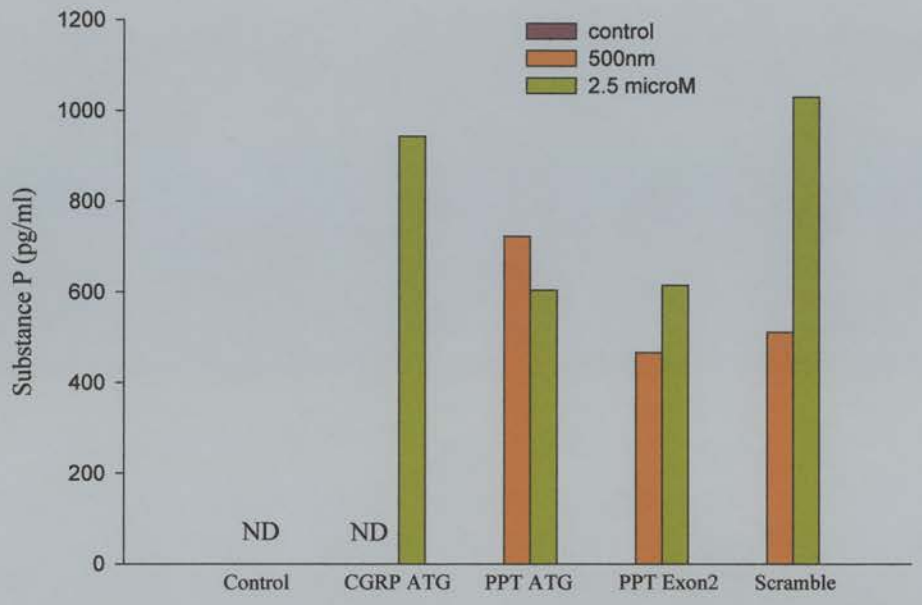
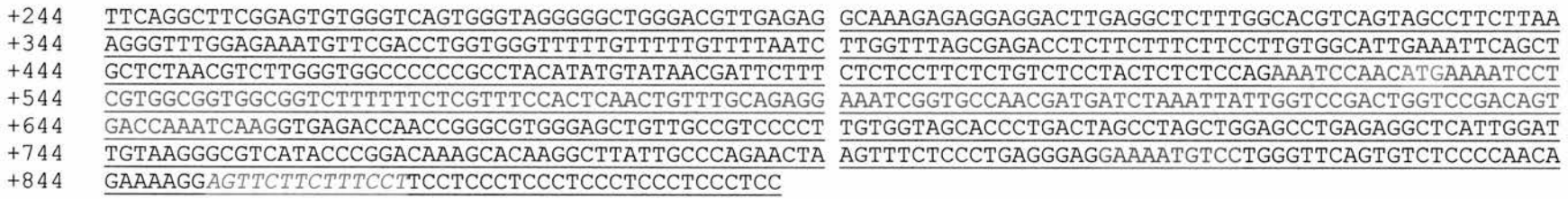


Figure 5.2 Effect of application of phosphorothioate antisense oligonucleotides, at 2 different doses, on production of SP by adult DRG neurones. Experiments conducted in 4 cultures per group, but some levels below detection limit of the assay (400pg) so not included in graph. ND= no data (all values below 400pg), CGRP ATG 2.5μM n=4; PPT ATG 500nM n=2, 2.5μM n=2; PPT Exon2 500nM n=3, 2.5μM n=3; Scramble 500nM n=1, 2.5μM n=4.

5.2.2 Identification of putative transcription factor binding sites in the promoter regions of rat βPPT-A and the α-CGRP encoding gene

The sequence of the promoter regions of both these genes were obtained from the MRC HGMP database. The βPPT-A promoter, spanning bases -3355⇒+893 relative to the transcriptional start point (accession number L07328), and the α-CGRP promoter, spanning bases -1366⇒+3 relative to the transcriptional start point (accession number M34090), were analysed for the consensus sequences of GAS, ISRE and NFκB binding sites (see 1.14 and 1.15) using Gene Jockey II sequence processor computer software (Biosoft, UK). The results are displayed in figure 5.3 (βPPT-A) and figure 5.4 (α-CGRP).


-3356 GGATCCTGCTCTCCCTGGGAGTAATTTCTCAAAGCAAACTATTTTGTTCT ACAATGTTTGGTTTTTCACTTTTGTAGAGTACTGTAGAAAAATGATCTGA
-3256 TGACTTTTTCTCTAAAGTTGTAAGCAGTATAAAAAGCTTTAAAGTGACTT ATGTTCAGTCACATGAAAATGGACAATAACATCAGTTTAGTTTTCTTTTTT
-3156 TAAGGTATATGTTTTTTATTATTCATGTATAAATGTTTTGCATGTGTTTA TGTGTAGACCTTGTTCATGAAGCTCTCAAAAAGGACAAAAGAGGGCAGCA
-3056 GATCCCCTGGATTATAGATTACGAATGGTTGCCAGCTACTACATGGGTGC TGGGAACTAAACCCTCATCTTTAGCAAGAGCAACAAGTACTTTAACCCCT
-2956 GAGCTATCTGCCCAGTCCCTCTTTATAATTTTCTACGGATAATTAATTGG CAAAAATGAAAATATGATACTTTGTTCACCACTCTTGTTCCAATGAACTT
-2856 AGTATGCATCAGGAACACTTGTATTTTATTAAGATTTGTATTTTAAACAT CTGAGGAAATGCATTAATTAGTAATACAAATATCTTCAGTCCTGCTATGT
-2756 TCAAGAAAAACATGAGTCAGAAAGTATATGAAATTACTAAGAAAATTAAG GAGTATTTAATGATGGTCCTGGGCAAGTGGGAAGTTAGTTGGGTACTAAC
-2656 ATTGACTGACCTTTGGTTAAGATCTCTAAGCAGACAGCATCACAACTATT AGGACACATTCAAGAGGAAAGAGGAAGTGATTGCATCAAAGCACCAGAAA
-2556 ACAACTCTGAGTGAGAGCCTGCTGCTTTGCAAAACAACCTCTCCCAAGAA CTAAGCAGAAGTCCCACAAAAACTATGCCAACATCTTCCAAAGCCTGCAC
-2456 CCTGATGACTGCTATACCAACACTAGAATGTCTCCATTAGATGTCTGGCA ACTTATCAACATGGCCACATTGATGACTAAGTGTGCAGTCCATGAACTGA
-2356 GGGGAAACAAACCACATTCAATCTCCAGCAAAAGGAAAATGTTTAGCAGA AGGAAGAGAAGGAAAAACGAGGCCTGAAGCTATGGTCCTTTTAATATATT
-2256 ATTATTAAAGATTAACATATATATATGATTAAAGATTGCCTTTTAGAGTT GTTCATATTATATAACTGTAAGATTAAATTAAAAACAATTTATAAATTTA
-2156 AAAGCAAAATGATCAAATAAGGTTTGGCACCATAACTACTTACAGACATT TTAAAAATAATTTTTAAGAAGTGAGGTACCTTAAGTGAACATACCTACAG
-2056 TGTTATGCCCCTAAAGTCAGAAAAAATGCAAAAAAAAAAAACCCTATTATC TAATTCCAACATGAATTGTTGAGCATGTTGACAATTGAGTGAGTGGCCAT
-1956 GTGAATGATATGACTGGCAAGTGGGAATTTTGGTATTGAAAGTGTCATGA AATATACTAGGTTAAATAAAAGCCATGATATTCCGATTGTTTAGAAAGTG
-1856 TCCACATGTTTGTGATTTTATATACTACACACACACAGAACATTAACACA TACTTATTCGTACACAGACACATTCTCTCTCTCTCTCTCTCTCTCTCTCT
-1756 CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCCAGCACCATGCAT TACCTTGAAGCAATGAAAATGAAAATGACTCCTGGAGTCTTTCGTAGGAA
-1656 ACATCAAAACCAAGAGAACCATCAATTCCTGTCAAAGGCCTTGGAAGAAT CTTCTGATAGCCAGGAATGGCATAAGTTGAGCATCAGTTAAAGAGTATCT
-1556 TCCTTGCACTGAAATGTTAAATCTGTGAGGTTCTAGTCTTAAAAAAGCAG GTCATACTTAAAAAGTGCTAAATTAGTGATTTTTAAAGTTAGTAGAGGGA
-1456 AGAAATCAGGGATTTAGCTCATATTTCCTGAATTATGTATCAGAATAACT ACACAGTGCATGAGAAAATATTTTCTATGTAAGTGGAATCCTGAAATTGA
-1356 CAAATTGTTTTAGGCGAATGACTCTACATTTGTGTTCACAATTTCTTCTT TTAATGGTTTTGAAAATTAAAAATAGAATGTTCAGATTATGAAATCCATC
-1256 TGAATGTCTACATTCAGAAATAAAAACAATAAACATTGGAGGCGAACATC AGTTTGGCTCACAAACACTTCTTTCCTCTTCAAATTATACTAAACATAGT
-1156 GGGAAACTATGATTCCCCAAGGAAAACAACTCTATCCAAATGACAGCATC ACTCGATGTGGTGGTCCACACCTGTAGTTCCAGTCCTGAGTAGGCGGAGT
-1056 CAGGAGGATACCCAGTTTGAAGCCACATAATGAGACTTAGTAATAATAAT AATAATAATAATAATAATAATAATAATAATAATAATAGCAACATCACTTA
-956 CTTCATCAAAAGATGGAAAAGAATTATACAGTGGGGACAGAAGGCATCTG TAAAACCATGGTTTGTAAATGACTTCTGTCTCTGACAGATCTGCAGAGCT
-856 CCAAAGGTAAGCATCCAGCCTTTCTAGTCCCCCAACAAGGCTAAAGGGGA GAGAGGCACAATTATCCTCTTCCCACCCCTTCTGCCTTCAGGGTGTGCCT
-756 GGGAAGAAGCTGTAGGGGAACAAAAGATGCCTTAGAATGGCTGATGGGTA AGTTCTACATGAGAAAGGAGGTTTAAATTCCTCTTTCCCCTAAATGTAAA
-656 ACAAACCTGCCTTCATCCTCTGAAGCGGGAGACCGGAAACACTTTTGCAG TGCTAGAGAAATGAGAATATTCTGACTGATTTGGTGGGGAGGGGGGTTGG
-556 GGGGGTGTGTTCCAGCCCTAGATATAACACCTCATAAACCTTAAGACACA TAAAGTAGAAATGAAAGGAAAACCCCGCTTGCTTCATCCCTCTGAAGTGC
-456 TTGCTGGTGTCTTAGTATTATTCACAAGGTTTTGCTGCTCAAGTTATTTG GCTGTCCTCAAAGCGCAATATTCCCTGATGCCTCTTGAGAGAAAAGTTCC
-356 CTAAGTCCGAAGCATGAGTCACTTCGCTCAGTTTTGATGAGTAATCTCAG GTGTCACTGAACCTTGTTCGGAAGAAGAGGGGAGGGGGGCGTCAGATTTG
-256 CAGACGGAAGAAAACAGGTCTCTCTGGATTGGATGGCGAGACCTCGACTTCCCTAAAATTGCGTCATTTCGAACCCAATTTGGTCCAGATGTTATGGACT
-156 CCGACGGGTTACCGTCTCGGAAACTCTATCACGCAAGCAAAAGGCGAGGG GGCGGCTAATTAAATATTGAGCAGAAAGTCGCGTGGGGAGAGTGTCACGT
-56 GGCTCTCCAGGCTCATCACGCCTGAGATAAATAAGGCGAAGCAGGAGCAG GGACTAGAGCGCACTCGGACCAGCTCCACTCCAGCACCGCGGCGGAGGAG
+44 AGCGAGGAGCGCCCAGCAAGTGCGCACCTGCGGAGCATCACCGGGTCCGA CCGCAGTGAGTACCACTCCCGCCTGCCATCTGCCGCTGCCTGGGGCCCGT
+144 CGTGCCTTGGTCAGAGGTGGCGTCCGCCCGGGGTTTCACCTGCAGCAGCC ACAGACCCGGAGAGAAACTGTGCAAATACCAACACCTCTCTTTGTCTGCC



Key	***TTNCNNNAA***	GAS site
	AGTTTCNNTTTCN$^{T}/_{C}$	ISRE (1 base mismatch)
	AGTTTCNNTTTCN$^{T}/_{C}$	ISRE (2 base mismatches)
	GGGA$^{A}/_{C}$TN$^{T}/_{C}$CC	NFκB binding site (2 base mismatches)
	Underlined bases	DNA transcribed to mRNA
	ATG	Start point of protein translation
	Blue bases	Exons

GAS	-3331⇒-3323	NFκB (2 base mismatches)	-1933⇒-1924
	-3249⇒-3241		-1052⇒-1043
	-2575⇒-2567		-593⇒-584
	-1432⇒-1424		-490⇒-481
	-671⇒-663		-212⇒-203
	-207⇒-199		-57⇒-48
ISRE (1 mismatch)	-3172⇒--3159		+814⇒+823
ISRE (2 mismatch)	-680⇒-667		
	+853⇒+866		

Figure 5.3 Promoter region of βPPT-A encoding gene analysed for putative transcription factor binding sites

-1366 CTGCAGGAGGGACTTGAAGGCAGGCTTGGGACAATTTGAGAATGAACCCC TAAGGATGCTTCTGTGGGCCACAGAGACTGCTGAGTGGCTGTGCTTTCTG
-1266 GATACGGTACCATTTTGGAAAGAGAGGAGAGTCTCTGTGCCAGGAGAAGT GTGACTGGTAGTGAATGTGAGGTTTAGTACGGGGCAACATCTCCACAGCG
-1166 CTGTCAAGCCTGCCTGCCTGCTCTTCAGCTCTTTAGCTCGGAGATCTAAG GGTGGGGGTAGGAGGGGAGCCACCGGACCAAATACAACTGGGACATCTTG
-1066 GCAAACAGCAGCGGGAAGCAAAGGGGCAGCTGTGCAAATCCTTAGGCAGG CGGGCGGGCGGGCAGGCGGGCGGGCGGGCGGGCAGGCGGGCAGGCGGGCG
-966 GGCAGGCGGCCGGATGAGTAGTGATGGATAGCCAGGCAGGAGGTGGAGAG ATCTACACTGGAGACTTTAGAGGCATCTGGTCCTTCCTCACACTGTCCCC
-866 ACTACCCCGTACCCCTACTCCCTACCCCAAGCAGGACCCAGCTGAATACA ACCCCTTCTCACACATGTGAGTGAGTGAGTTATCCAGCACATAAGAATGC
-766 CAAGCTGAAGACGGATGATTCACTTTGGGGAAGGAGAGATTTTATAGCTC AGGAAACACCAAGGTTTCTGCCTACTAGCCAGGCCCTTCAAAAGGGGACC
-666 AGGATACCCACTGAAAAGTTTAATATGTTGAGCTTTCGTGCAGGCCTTTG GGGGTTTGGGGGGGGGGGAATTTTGA*ATTTTTTTTTTCGT*TTTGTTTTTA
-566 CCTGTGGTCACATAACCAGCACGAGGCAGCTACAAGGTTCAGGTCTGACA GAGCCCCTGTGTCCAGCACCAACACCTTTGGCTATCAGCCTAAACCTGTG
-466 CCACCCTGCCAAAGCCAGCCTTGCAGACCAAGAGTCCACCCCTACGGTGC ACTAAAGTCTTCCGGATTAGGCACGGACTAGGGTCGGGGCACGATTAGAA
-366 TCAGACATGCAGCAAGGAGTACTTGAGATACTGGACTCTACTCTCCAAGG TCCAGAGATTGGAGTCGGGGATGTTCAAAGTCAGGAGGGAAGAAGAGATA
-266 AAATTTACCTTGACGTCAAAAGGCCCTCCAAATTCCCGCTAATTTTAAGG GTGGTTCTCACTGCTCCCCACCATCCTCCCAC***TTCCATCAA***TGACCTCAA
-166 TTTAAATTCAAATGGTGTCATCTTGCTAGATGCTCGGAGTTCTGGAAGCA CCGAGGTGACGCAATCTGTCTGGGGCACGGGGCCCTTCCACCTATTGGCT
-66 GCCTGGCGCCCCGGGACCCCTCCCAACTACCGCGGCGGGAATAAGAGCAG CTGCAGGCGCTTGGAAGCA

TTNCNNNAA	GAS site
AGTTTCNNTTTCN$^{T}/_{C}$	ISRE site (2 base mismatches)
GGGA$^{A}/_{C}$TN$^{T}/_{C}$CC	NFκB binding site (2 base mismatches)
Underlined bases	DNA transcribed to mRNA

Putative Binding sites	GAS	-183⇒-175
	ISRE (2 mismatch)	-589⇒-576
	NFκB	-601⇒-592
		-335⇒-326
		-87⇒-78
		-54⇒-45

Figure 5.4 Promoter region of α-CGRP encoding gene analysed for putative transcription factor binding sites.

5.2.3 IκB-α in L5 of untreated and inflamed animals

Animals received an ipsilateral intradermal injection of FCA around the joint (as described in chapter 2.1) 30 min. before sacrifice. IκB expression in protein extracts from L5 DRG ipsilateral and contralateral to the injected limb was examined by western analysis.

The antibody used in these experiments was specific for IκB, displaying no cross-reactivity to recombinant p65 (figure 5.5). Bands detected in DRG extracts were of a similar mobility to recombinant IκB-α, with an approximate molecular weight of 40kDa. The Mw of IκB-α is cited as 37 kDa (Israel 1995), therefore it seems likely that IκB-α is present in all DRG samples.

As shown in figure 5.5, there is no decrease in intensity of IκB-α bands taken from untreated DRG or those from animals 30 min after FCA injection, indeed there appears to be a slight increase in band intensity. Thus it would appear from these experimental results that degradation of IκB-α is not apparent in DRG 30 min after adjuvant administration.

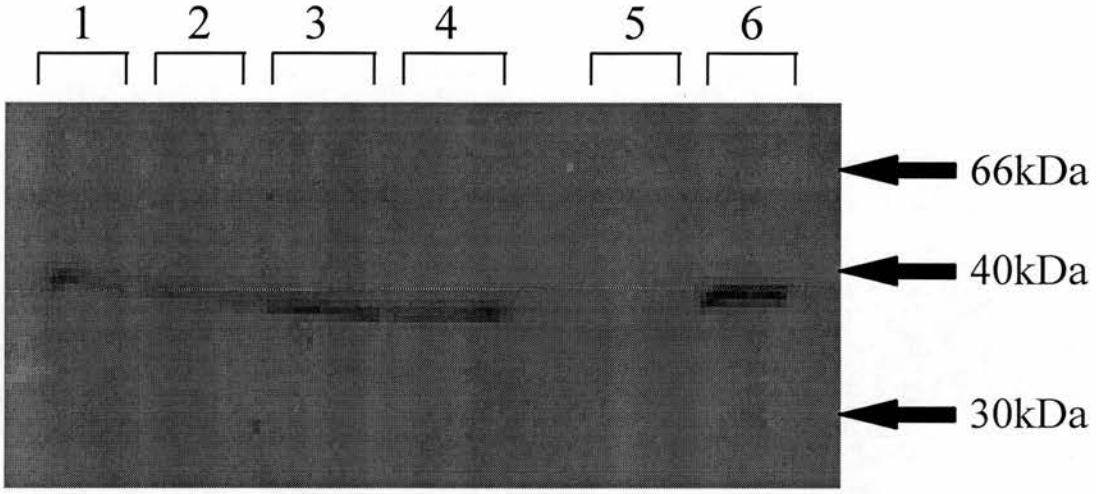


Figure 5.5 Western blots using an antibody specific for IκB-α and extracts from L5 dorsal root ganglia from rats treated as follows;

Lane 1 Untreated animal

Lane 2 30 min after contralateral FCA injection

Lane 3 30 min after ipsilateral FCA injection

Lane 4 30 min after ipsilateral FCA injection

Lane 5 Recombinant p65 (negative control)

Lane 6 Recombinant IκB-α (positive control)

5.2.4 Analysis of STAT gene expression in DRG by PCR

To analyse STAT gene expression in DRG, degenerate primers, designed to amplify STAT 1,3 and 4 (C. Watson, personal communication) were used in RT-PCR experiments 1μg of total RNA isolated from L5 DRG and liver were reversed transcribed. Resulting cDNAs were separately subjected to 30 cycles of PCR (section 2.11) using STAT primers or primers designed to amplify β-actin, as a positive control. Primers designed to amplify β-actin produced the predicted 550 bp product in both DRG and liver samples (figure 5.6). This demonstrated that cDNAs had been successfully produced by reverse transcription.

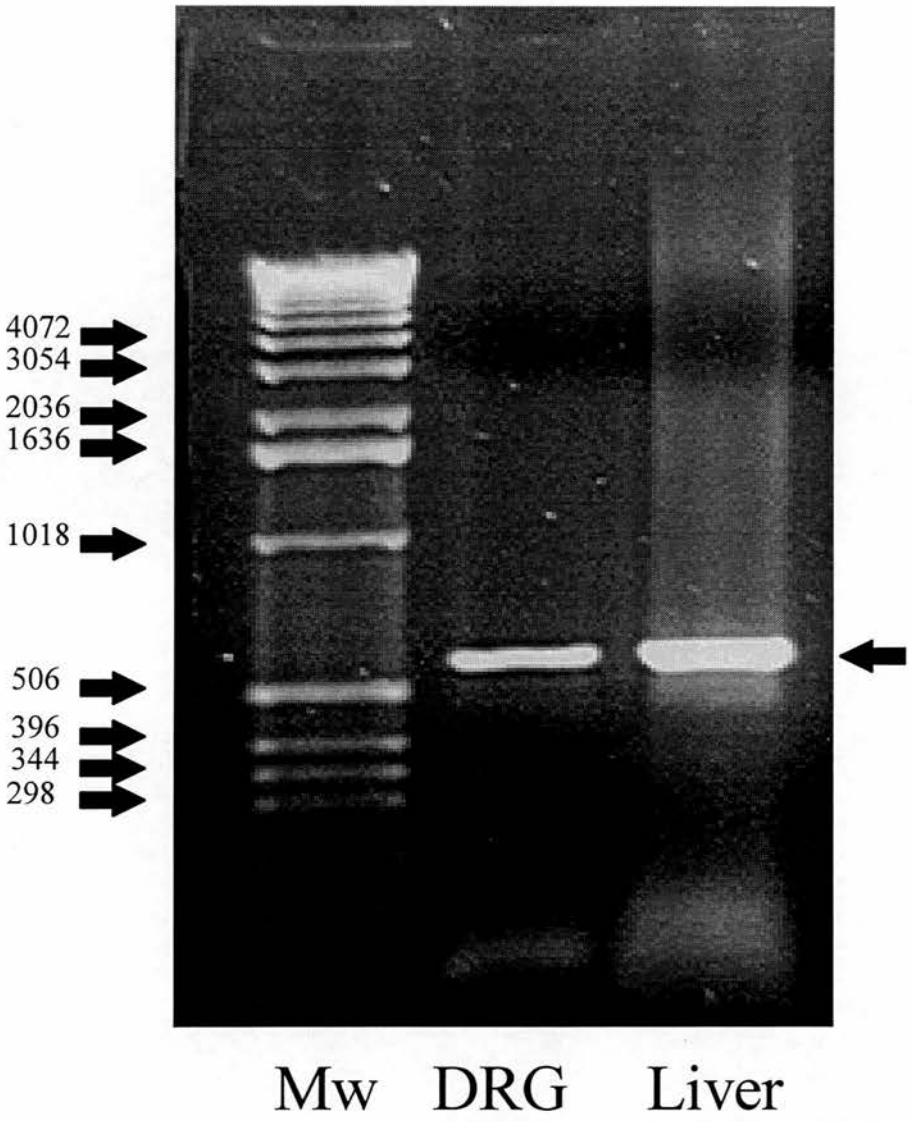


Figure 5.6 Bands representing specific polymerase chain reaction products from β-actin primers and cDNA from dorsal root ganglia (DRG) and liver. Bands in the lane labelled Mw represent molecular weight markers (1Kb Ladder, Gibco BRL), sizes in base pairs are given to the left of the image.

The degenerate STAT primers produced a single DNA fragment in both liver and DRG of approximately 450 base pairs (figure 5.7).

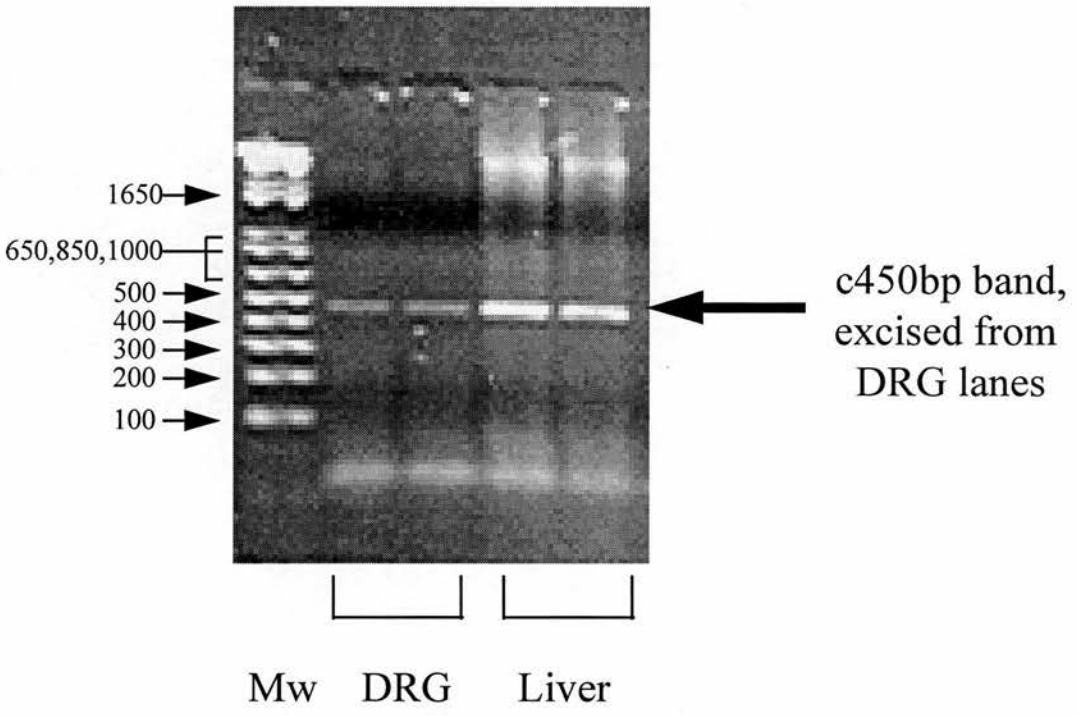


Figure 5.7 Bands representing specific polymerase chain reaction products from degenerate primers for the SH-2 domain of STAT 1,3,4 and cDNA from dorsal root ganglia (DRG) and liver. The bands in the lane marked Mw represent molecular weight markers (1KB Plus Ladder, Gibco BRL), sizes are given in base pairs on the left of the image. The two lanes marked DRG and Liver are taken from a single reaction

The PCR product generated was subcloned (chapter 2.11) to allow partial DNA sequencing of this product, detailed in table 5.3. The sequencing was performed by Val Lyons.

```
  1  GGGCTGCTGG TCCTTGAGCA GAGCACGTTC TCGCTCCTTG CTGATAAAGC
 51  CCATGATGCA CCCATCATTC CAGAGGCAAA GCAGGTGCTT CTTAATGAGT
101  TCTAGGATGG TGTCAATCCA AGGCTAGAAG GAGAAATTCT TATCGTTAAT
151  ATTTTCCTTA CAGAACCTTG TCCATGGAAT AAGACCATCA GGGCCA
```

Table 5.3 Nucleotide sequence of PCR product produced from DRG derived cDNA and STAT primers

The nucleotide sequence obtained was used to search for similar DNA sequences in the Genbank and EMBL databases. The results are presented in table 5.4.

Member	Species	Base Matches (% identity)	Accession Number
STAT 1	Mouse	184/195 (94)	U06924
	Human	152/175 (89)	M97936
STAT 2	Pig	62/92 (67)	AB004061
	Human	51/79 (64)	U18671
STAT 3	Rat	111/163 (68)	X91810
STAT 4	Human	113/165 (68)	L78440
STAT 5a	Rat	91/157 (57)	U24175
STAT 5b	Mouse	84/144 (58)	Z48538
	Human	92/158 (58)	U48730
STAT 6	Rat	82/142 (57)	AF055292

Table 5.4 Results of the blast search of the PCR products produced from DRG cDNA using degenerate primers for STAT 1, 3 and 4.

5.3 Discussion

Results from the AS-ON studies suggest that the phosphorothioates oligonucleotides are having a 'stress effect' on cultured sensory neurones, causing a dose-related

increase in production of SP and CGRP. In subsequent work utilising DRG cultures Mulderry and Dobson demonstrate the inhibition of c-Jun expression by microinjection of phosphorothioate oligonucleotides (Mulderry and Dobson 1996). This was reported to not alter the number of cells immunopositive for SP, the effects on total SP levels in DRG are unknown.

In experiments to elucidate the role of the low affinity neurotrophin receptor, p75, in sensory nerve survival, phosphorothioate AS-ONs have been successfully employed to decrease receptor number in cultures of DRG neurones (Barrett and Bartlett 1994). However unlike the experiments presented in this chapter, AS-ONs were mixed and triturated with the cells immediately before plating. Furthermore these AS-ONs were also effective *in vivo*, being retrogradely transported to DRG cell bodies after application to the proximal stump of a transected peripheral nerve, and decreasing the number of p75 immunopositive neurones (Cheema et al 1996). However, this method would be unsuitable for the analysis of SP and CGRP after FCA induced joint inflammation, as nerve transection itself causes a decrease in the expression of SP and CGRP (Hokfelt et al 1994).

Phosphorothioate oligonucleotides, regardless of size, induce expression of the transcription factor Sp1 in a wide variety of cell types, this induction was blocked by inhibition of the NFκB (Perez et al 1994). Thus, phosphorothioate oligonucleotides may stress the cells resulting in NFκB induction, which in turn increases the expression of target genes. It is possible that such a mechanism may account for the non-specific effects seen here.

A number of kinases have been implicated in IκB phosphorylation. These include PKR (Kumar et al 1994), also referred to as double-stranded RNA activated protein

kinase. As the name suggests the kinase is activated in the presence of double stranded RNA (dsRNA) and phosphorylates serine and threonine residues (Meurs et al 1990). dsRNA is found in virus molecules, and so PKR has been implicated in an anti-viral role. However, the mode of action of antisense oligonucleotides is thought to be dependent on the formation of double stranded species between interaction of hnRNA or mRNA and the applied AS-ON, thus AS-ON could result in PKR activation. PKR has also been shown to phosphorylate IκB-α *in vitro* (Kumar et al 1994), thus potentially activating NFκB dimers bound to IκB-α. The activation of NFκB would then result in increased expression of genes with the ability to bind this transcription factor.

The original aim of these experiments was to establish a method of selectively decreasing SP or CGRP expression in DRG neurones and hence determine the involvement of these neuropeptides in the aetiology of FCA induced inflammation. Since these experiments were carried out the role of SP in inflammation and pain transmission have in part been answered by studies using animals in which the PPT-A gene or the gene encoding the NK-1 receptor had been disrupted. The validity of results obtained from these knockout animals are discussed in section 1.14.

In view of the activation of NFκB by phosphorothioate oligonucleotides and the increased production of SP and CGRP in cultures of DRG neurones treated with phosphorothioate based AS-ONs the promoter regions of both βPPT-A and the gene encoding α-CGRP were analysed for NFκB binding sites. A number of sequences with limited homology to the consensus binding site for NFκB were found in the promoter region of both genes, however it is not known if these sites are capable of binding members of the NFκB family. Experiments utilising specific antibody

showed that IκB-α is present in DRG, although it is not clear from these data which population of cells contain IκB-α. The presence of IκB-α infers that members of the NFκB family (p50 and p65 being the most likely) are probably also present in DRG.

The original aim of this experiment was to use the levels of IκB as an indication of the activity of NFκB. However IκB gene expression is positively regulated by NFκB (Sun et al 1993), therefore NFκB activation leads to *de novo* IκB production, replacing IκB degraded as described in figure 1.6. In previous experiments I have shown that there are increases in DRG SP and CGRP levels within 30 min of FCA injection. If expression of these peptides is regulated by NFκB it is likely that IκB expression would also increase.

This indicates a limitation of this experiment, it would have been better to investigated using electromobility shift assay (EMSA). This technique involves the ability of protein extracted from nuclei to bind to a synthetic oligonucleotide of a transcription factor binding site, and thus the activity of the transcription factor of interest. However, because of logistical problem in the small amount of nuclear protein obtainable from DRG and the relatively large amounts needed for EMSA analysis, these experiments are technically difficult to perform in this model. IκB-α is the commonest of the IκB family, however this experiment does not take into account other members of the IκB family, which may be present in DRG cells and play a role in NFκB activation.

Immunocytochemistry studies investigating the active form of the NFκB family member, p65, reported this transcription factor to be found in all diameters of L4 and 5 DRG neurones (Doyle 1997). The highest distribution was in cells of small

diameter, the size of neurone associated with transmission of nociceptive stimuli, and with the highest distribution of cells expressing PPT and CGRP. Interestingly the number of cells immunopositive for the activated form of p65 was greatly reduced within 4h of sciatic nerve crush (Doyle and Hunt 1997), a condition that is also associated with a decrease in SP synthesis (Nielsch et al 1987, Hokfelt et al 1994), suggesting a possible role for NFκB in the regulation of PPT.

A region within the PPT promoter, between bases +92 to +500 has been identified as a major enhancer of promoter activity in DRG under basal conditions (Mulderry et al 1993 a). This area shows a large degree of homology with an area 5' of the transcriptional start point between bases -700 and -500, which also has enhancer activity. These two similar areas contain an AT rich region which can be bound by members of the NFκB family and also members of the octamer binding protein family as demonstrated by EMSA analysis (Fiskerstrand et al 1997). These experiments were conducted *in vitro*, therefore it is difficult to draw definitive conclusions about their role *in vivo*. However NFκB binding has been reported in DRG extracts 6h after inflammation induced by intraplantar injection of TNF-α (Wood 1995). The AT rich regions to which NFκB are proposed to bind to in the PPT gene, show little conservation to the consensus sequence of NFκB ($GGGA^{A}/_{C}TN^{T}/_{C}CC$), which may indicate a novel binding site for NFκB.

Injection of FCA causes an increase in neural activity over a similar time course to the increases in βPPT-A and α-CGRP mRNA expression. Increased expression of both these neuropeptide mRNAs is prevented by local anaesthetics (Chapter 4). These results suggest that depolarisation of DRG neurones may underlie increases in neuropeptide encoding mRNAs. Depolarisation in hippocampus by kainate

application causes NFκB induction (Unlap and Jope 1995) and also PPT-A expression (Brene et al 1992).

Taken together these data are inconclusive as to a role of NFκB in the upregulation of expression of genes encoding the sensory neuropeptides SP, NKA and CGRP caused by FCA induced joint inflammation. However there is enough circumstantial evidence to suggest it may be a fruitful avenue of further investigation.

Using RT-PCR the existence of mRNA encoding STAT 1, or a STAT 1-like molecule, in adult rat L5 DRG has been demonstrated. The form and cellular distribution of STAT 1 in DRG is unresolved, these questions could be addressed by immunocytochemistry. STAT 1 binds to DNA sequences similar to GAS, but not at ISREs. Analysis of the promoter region of both βPPT-A and the α-CGRP encoding gene reveals there are numerous putative GAS sequences in both genes, especially βPPT-A. However it is not clear if these sites are transcriptionally active, with the ability to bind STAT proteins The putative GAS sequence spanning bases -671⇒-663 of the PPT promoter is of particular interest as it lies within a region identified as an enhancer element by *in vitro* studies (Mulderry et al 1993 a). In addition there are sequences showing limited homology to ISREs in promoters of both neuropeptide encoding genes. It is possible that other STAT proteins are present in DRG.

Previous experiments have shown a number of cytokines and chemokines, which can signal via STAT proteins affect SP and CGRP expression in cultured sympathetic neurones (Freidin and Kessler 1991, Fann and Patterson 1994). One of these cytokines is interleukin (IL-6). The interleukin 6 receptor (IL-6R) has been located in DRG neurones of all sizes (Gadient and Otten 1996), and belong to a family of receptors which contain a common subunit, termed gp130 (Hirano et al 1994).

Receptors containing the gp130 subunit can signal via the Jak/STAT pathway and binding of IL-6 to its receptor causes STAT 1 translocation to the nucleus (Akira et al 1994). Leukaemia inhibitory factor (LIF) is one of the identified chemokines which causes an increase in neuropeptide expression in cultures of sympathetic neurones (Freidin and Kessler 1991, Fann and Patterson 1994). LIF receptors are also members of the gp130 receptor family and are thus capable of activating the Jak/STAT pathway (Hirano et al 1994). The effect of LIF on neuropeptide gene expression in DRG is unclear. LIF failed to alter SP or CGRP levels when applied to cultures of DRG neurones (Mulderry 1994), but reverses the decrease in SP-encoding mRNA in DRG caused by axotomy of the sciatic nerve, although only when applied at high dose (Zhang et al 1995). Upon intraneural injection into the sciatic nerve LIF is retrogradely transported exclusively to small diameter neurones, where it is co-localised with CGRP, and the high-affinity NGF receptor, trkA (Thompson et al 1997). However the observation that LIF increases during nerve injury (Banner and Patterson 1994) and that injection of LIF into the sciatic nerve and nerve transection induce galanin expression in similar populations L4 and 5 DRG neurones (Thompson et al 1998), have implicated LIF in axotomy, a condition associated with a decrease in SP levels in the DRG (Nielsch et al 1987, Hokfelt et al 1994).

LIF is hard to detect in the adult DRG under basal conditions (Murphy et al 1993), suggesting it is only expressed under specific conditions, this and the proposal that LIF is axonally transported from the periphery in the same manner as NGF (Thompson et al 1997), means it is not well placed to play a role in rapid plastic changes in DRG following peripheral inflammation.

5.4 Future Perspectives

Experimental data presented in this chapter have only scratched the surface of the possible molecular mechanisms underlying the FCA-induced increases in βPPT-A and α-CGRP encoding mRNAs. The class of transcription factors responsible for mediating such swift upregulation in the expression of βPPT-A and α-CGRP mRNAs are likely to be factors which exist in a labile state in the cytoplasm, and are rapidly translocated to the nucleus following phosphorylation at specific residues. The translocation of factors from the cytoplasm to the nucleus of L5 DRG neurones after FCA induced joint inflammation could be visualised using immunocytochemistry. However this approach would not identify the genes that were modulated by the transcription factors. Performing immunocytochemistry for transcription factors and in-situ hybridisation for mRNAs encoding sensory neuropeptides on the same or serial DRG sections may uncover a link between transcription factor translocation and gene induction.

Protein-DNA interactions, such as transcription factor binding, can be studied using a number of techniques including EMSA or DNAse 1 protection analysis. These techniques rely on the specific interaction between DNA (either fragments or synthetic oligonucleotides) and nuclear extracts derived from the tissue of interest. Theoretically nuclear extracts taken from L5 DRG at specific time points after FCA induced joint inflammation could be used. However with the technical difficulty of obtaining sufficient nuclear protein from rat L5 DRG to use in these kinds of experiments, this approach is likely to be of limited use. Higher yields of nuclear protein could be extracted from the DRG of larger animals, for example sheep.

However this would require the development of a FCA induced model of joint inflammation in the sheep which shows similar aetiology as the unilateral model developed in the rat.

Alternatively, the possible signalling mechanism induced by FCA could be the target of future experiments. Activation of signalling pathways is associated with phosphorylation of signalling components at specific residues. Antibodies which recognise the phosphorylated forms of signalling molecules could be used to gauge the activation of pathways in DRG neurones which show increases in βPPT-A and α-CGRP encoding mRNAs after FCA treatment, using immunocytochemistry and in-situ hybridisation. Phosphorylation generally occurs on serine, threonine or tyrosine residues, antibodies which recognise phosphorylated forms of these amino acids are readily available. These could be used in western blots to identify phosphorylated proteins by size if no antibodies were available specific for the phosphorylated form of a signalling molecule. Additionally, the antibodies which recognise phosphorylated forms of these three amino acid residues can be used to immunoprecipitate proteins phosphorylated in response to FCA induced joint inflammation. Western blots could then be carried out on immunoprecipitated proteins to identify putative signalling molecules.

Given that neural activity appears to be pivotal to the increases in βPPT-A and α-CGRP gene expression, the elucidation of the pathway underlying the depolarisation induced increases in PPT expression observed in cultures of primary sensory neurones (Mulderry et al 1993 b) would be of interest and relevance.

Studying the aetiology and plasticity in sensory neuropeptide expression following FCA induced joint inflammation in animals lacking specific transcription factors

may seem an attractive method of screening for potential mediators in the upregulation of βPPT-A and α-CGRP. However many transcription factors are widely expressed and play roles in development or in cellular processes in adult animals. For instance mice which lack STAT 2 or 3 die at the embryonic stage, whilst mice lacking STAT 4 or 6 have an impaired immune system (Darnell 1997).

If the loss of gene expression were more localised it may give a better idea of the potential involvement of various molecules in joint inflammation induced increases in neuropeptide encoding genes in encoding DRG. Once the problems of toxicity and effective delivery to the DRG have been overcome, AS-ON would be an ideal tool for attaining a localised block of the expression of specific genes.

With the identification of the pathway/transcription factor responsible for the increased expression of βPPT-A and the α-CGRP encoding gene it may be possible to devise a strategy for blocking this increase using specific inhibitors. Given the proposed role of the sensory neuropeptides in transmission of nociceptive information, development of hyperalgesia and neurogenic inflammation, this could be especially relevant in clinical cases of inflammatory joint disorders, such as rheumatoid arthritis.

6 CONCLUSIONS

Based on the results presented it seems the following sequence of events occurs in response to FCA-induced inflammation.

Injection of FCA around the tibio-tarsal joint causes rapid inflammation, as seen by the formation of odema and development of mechanical hyperalgesia with 30 min of injection. Within 15min of FCA injection into a joint there is an increase in the neural activity of innervating small diameter sensory nerves. As detailed in section 1.4, a number of mediators released in response to inflammation, caused by FCA injection, can excite or sensitise sensory nerves.

Within 30 min of FCA injection around the tibio-tarsal joint there are increases in mRNAs encoding βPPT-A and α-CGRP in innervating L5 DRG. These increases are prevented by prior administration of the local anaesthetic lignocaine around the sciatic nerve innervating the joint to be injected with FCA. Increased βPPT-A hnRNA levels 30 min after FCA injection indicate that the initial increase in βPPT-A mRNA is in part attributable to *de novo* gene expression. Taken together these results suggest that an increase in sensory neural activity causes increases in the expression of neuropeptide encoding genes in L5 DRG. These rapid changes in gene expression are too fast to be due to retrograde axonal transport of a molecule such as NGF from the periphery, or the *de novo* protein synthesis of a transcription factor. The latter hypothesis is supported by the observation that systemic injection of the protein synthesis inhibitor cycloheximide does not prevent FCA-induced increases in α-CGRP encoding mRNA. This evidence suggests that AP-2, which has previously

been implicated in playing a role in regulating an increase in both βPPT-A and α-CGRP encoding genes (Donaldson et al 1995 b), is not involved in the initial upregulation of these two genes.

The speed of increases in βPPT-A and α-CGRP encoding mRNA dictate that the increases in the expression of these genes are likely to be mediated by activation of a transcriptional modulator by post-translational modification, such as phosphorylation. Such a modification may be due to induction of a kinase cascade in response to the increased neural activity, and associated depolarisation of DRG neurones, activating a cell surface mechanism (receptor, voltage-gated ion channel).

A number of transcription factors are activated by phosphorylation (as reviewed by Jans 1995), these include members of the NFκB and STAT families. Analysis of the promoter regions of βPPT-A and α-CGRP encoding genes has found sequences showing homology to binding sites for members of both families. Other experimenters have identified two sequences within enhancer elements of the βPPT-A promoter which are capable of binding NFκB (Fiskerstrand et al 1997). Further investigations in this thesis have shown that members of the NFκB family and STAT 1 are present in DRG. Collectively these data suggest that members of the NFκB family and Stat 1 may be putative regulators of the rapid increases in the expression of the βPPT-A and α-CGRP encoding genes seen in response to FCA injection.

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